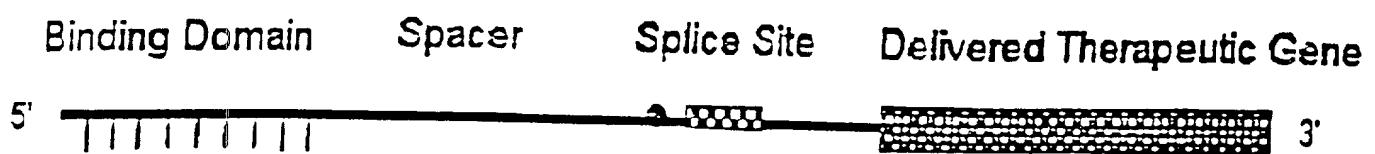
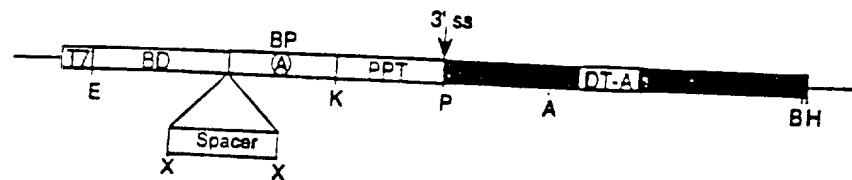


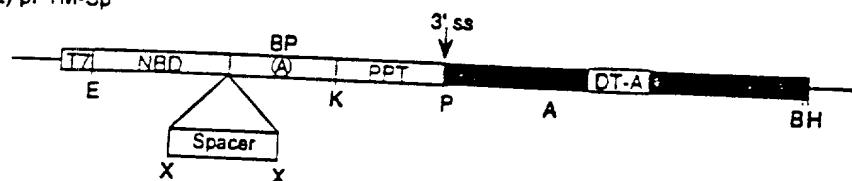
## FIGURE 1A.



(B) (1) pPTM+Sp



(2) pPTM-Sp



(C)

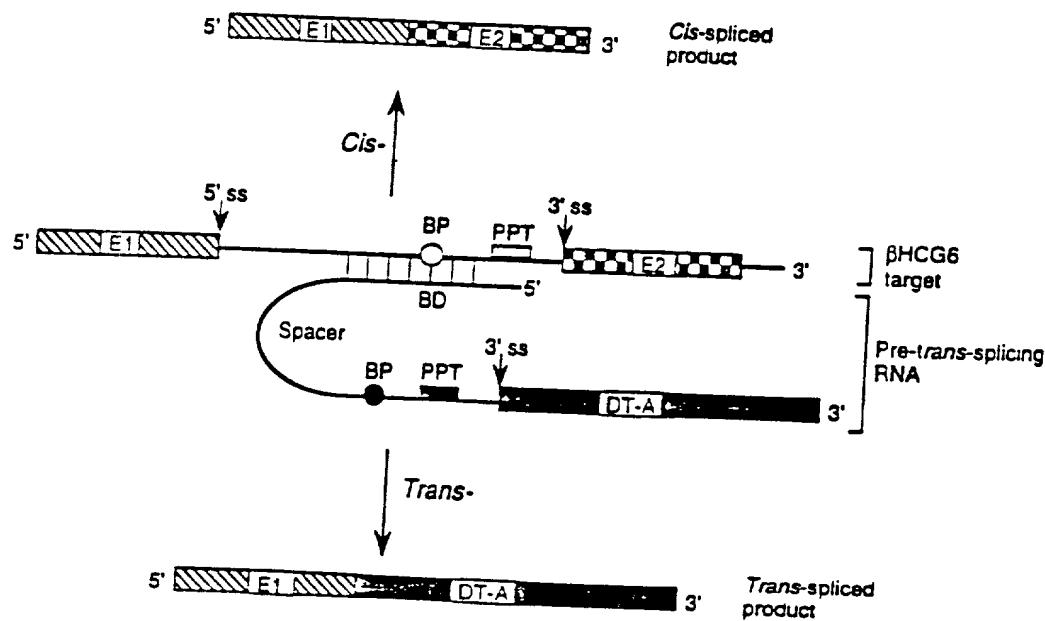
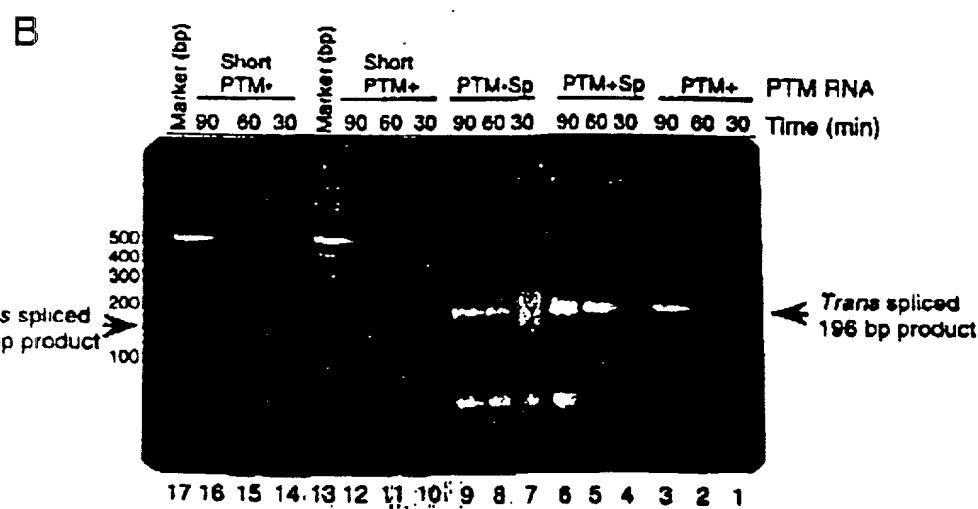
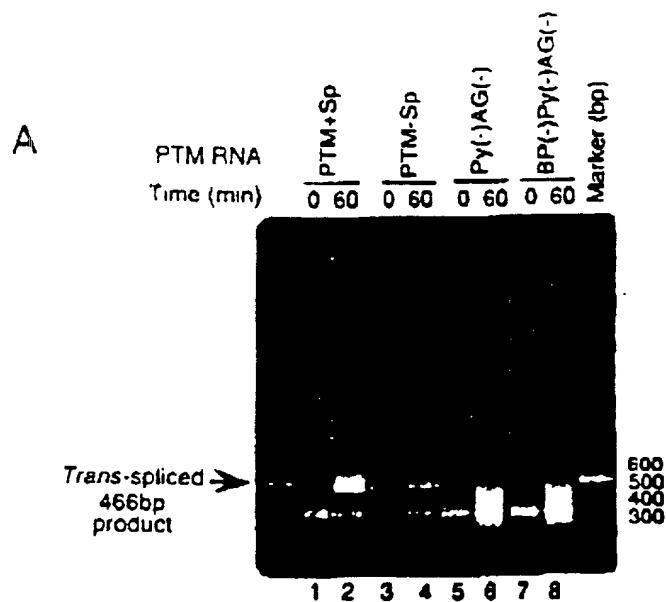


Figure 1 B-C



C T A G

Exon 1 of BHCG6

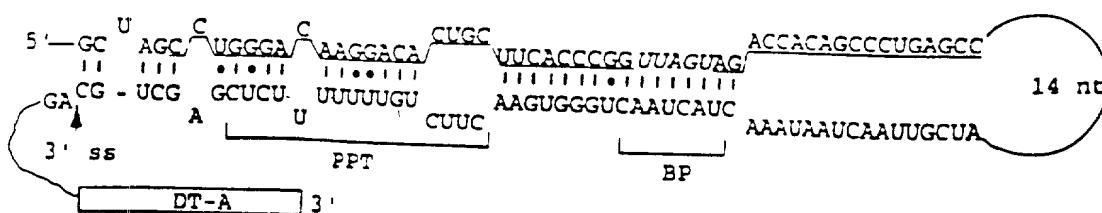
### 1st coding nucleotide of Rm +

G  
A  
G  
A  
T  
G  
T  
T  
C  
C  
A  
G  
  
G  
G  
C  
G  
T  
G  
A  
T  
G  
A  
T  
G

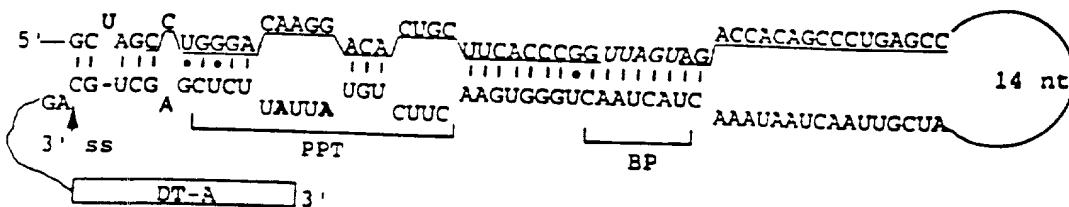
(A)

515045-A  
(Sheet 5 Of 5)

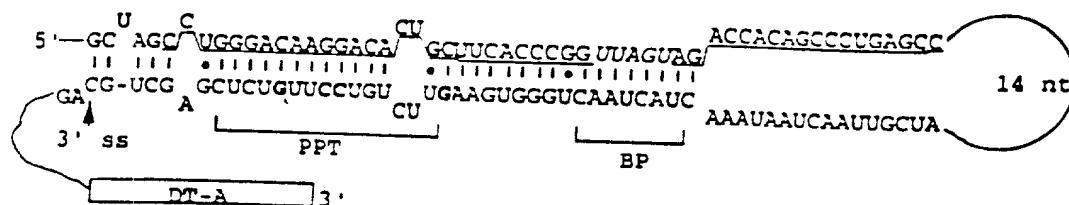
## 1. PTM+SF



## 2. PTM+SF-Py1:



### 3. PTM+SF-Py2:



(B)

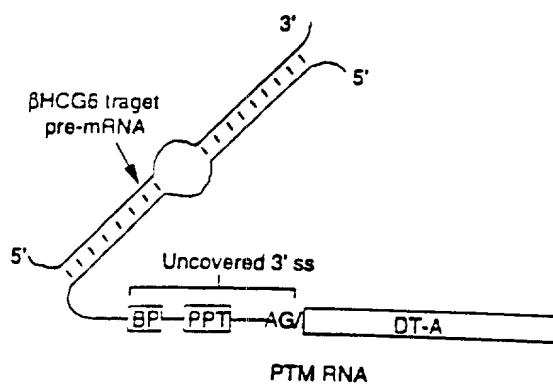


Figure 4 A-B

(C)

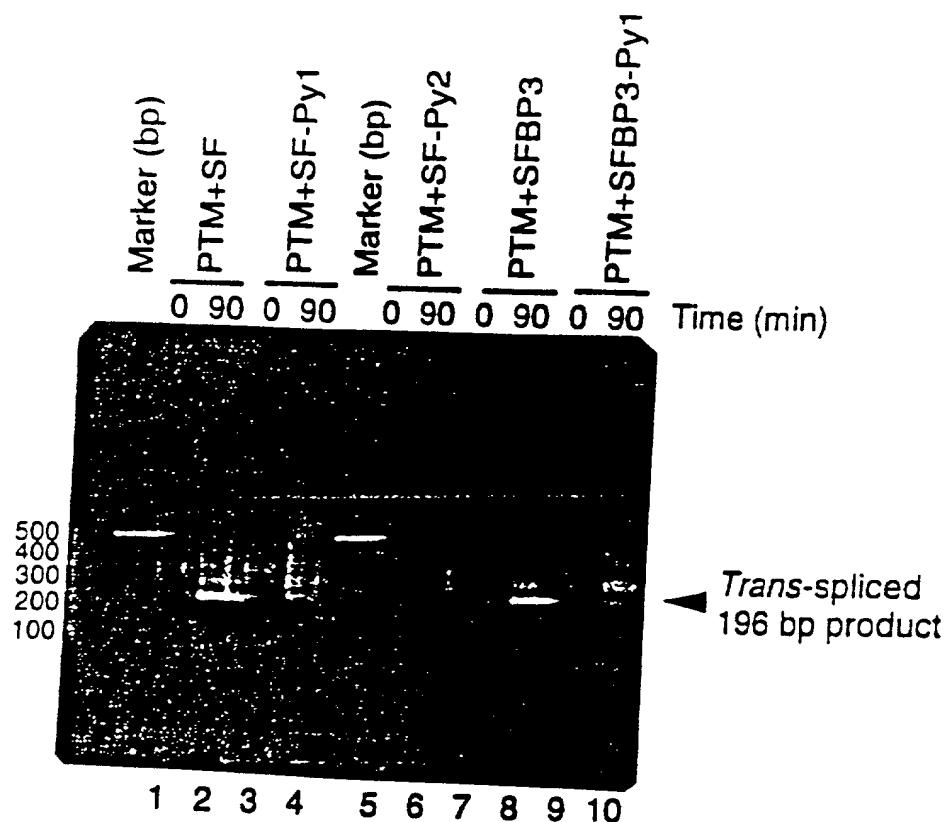


Figure 4C

31304B-A  
(Sheet 7 of 58)

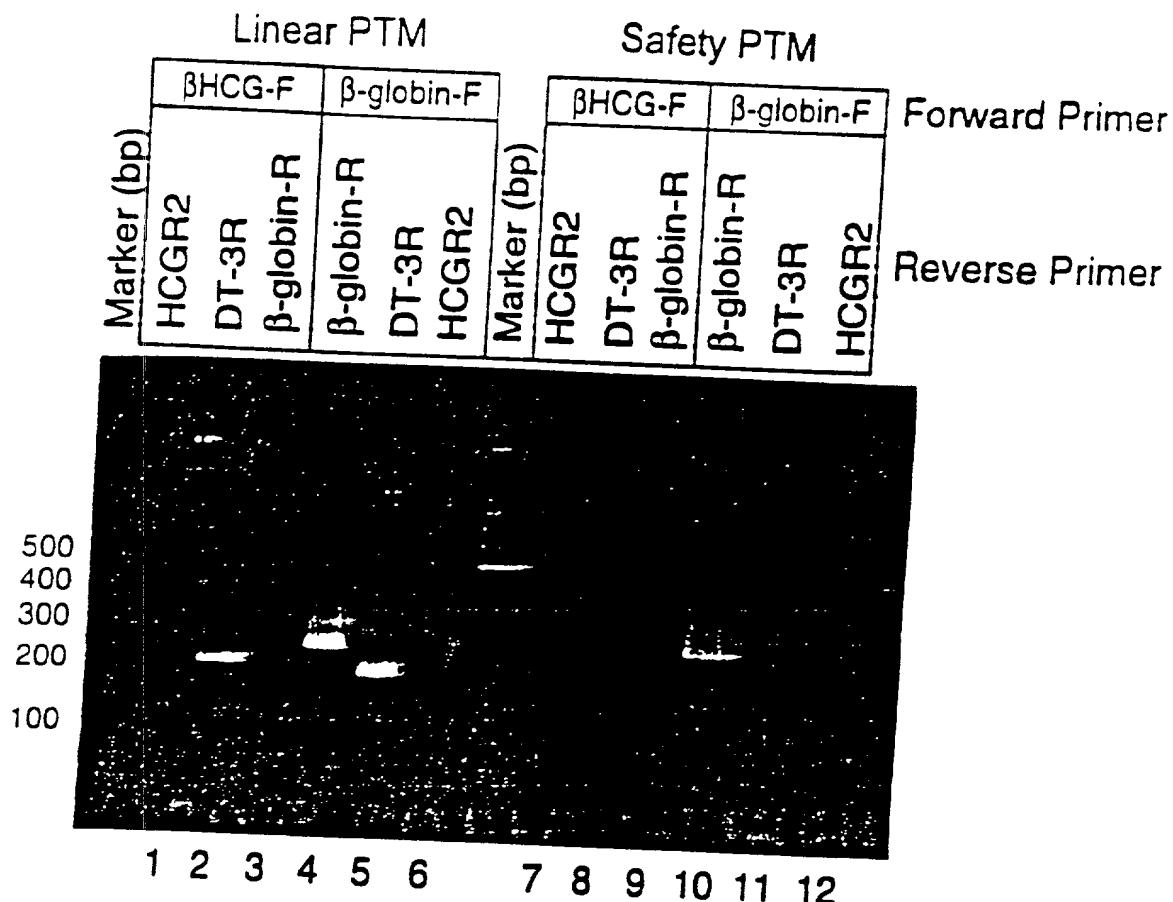


Figure 5

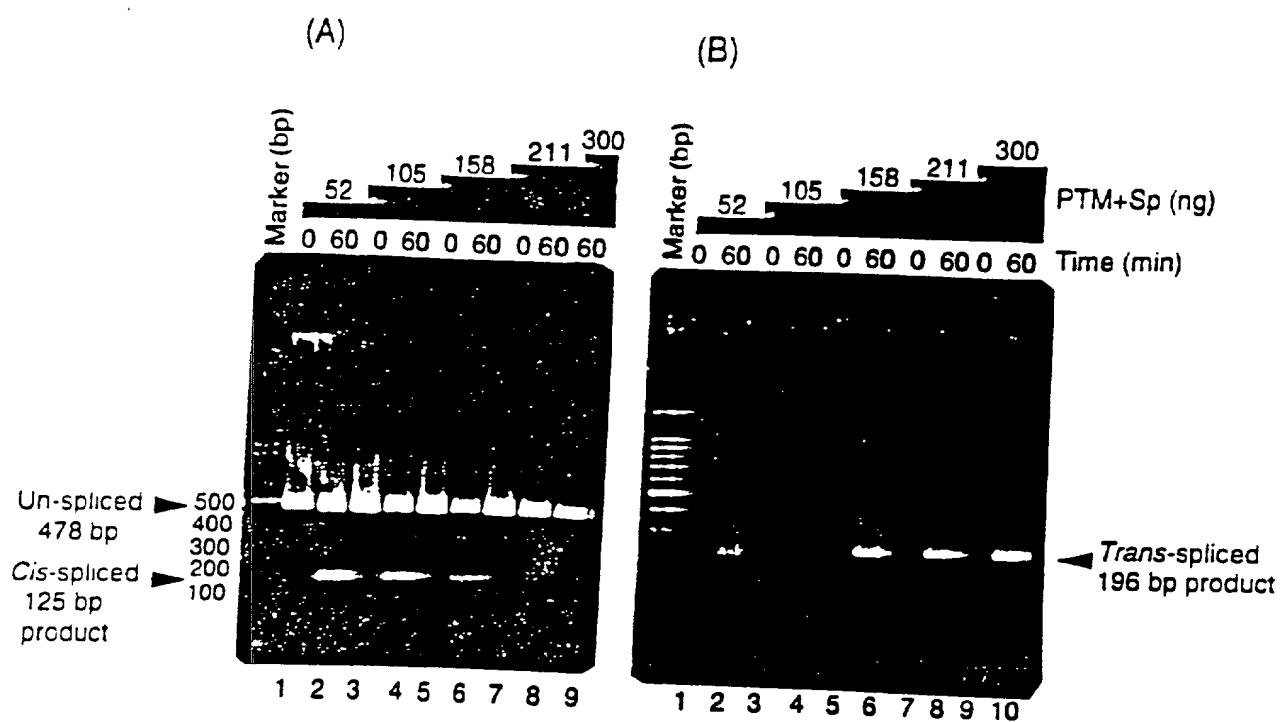
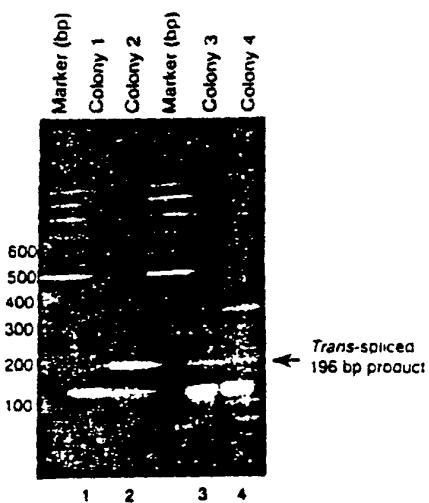


Figure 6

Figure 7

(A)

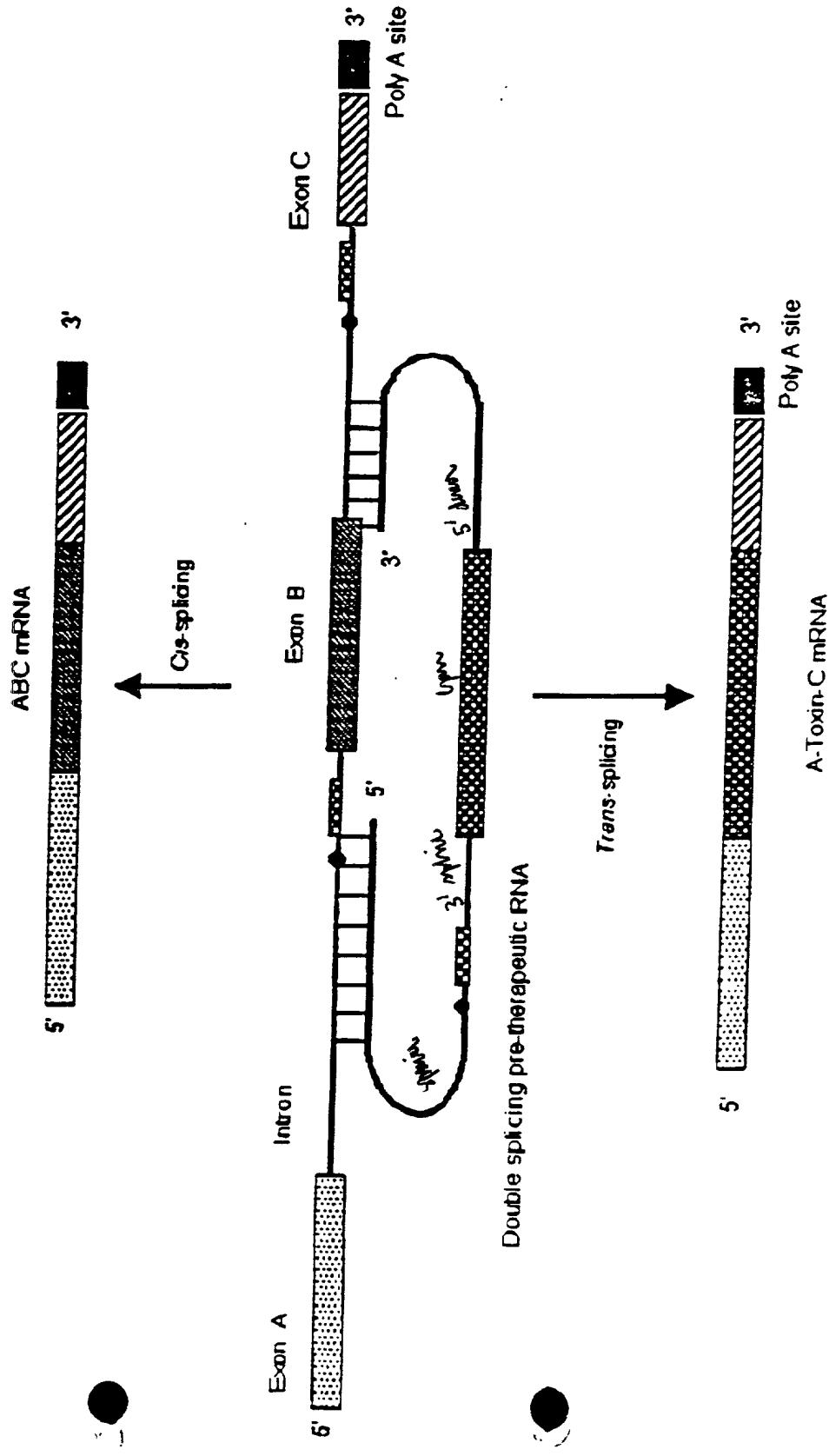


(B)

Exon 1 of  $\beta$ HCG6 ↓  
5'-CAGGGGACCCACCAAGGATGGAGATGTCCAG-GGCGCTGATGATGTTGTT  
↓ 1st coding nucleotide of DT-A  
GATTCTTCTTAAATCTTTGTGATGGAAAAACTTTCTTCGTACCAACGGGACTA  
AACCTGGTTATGTAGATTCCATTCAAAA-3'

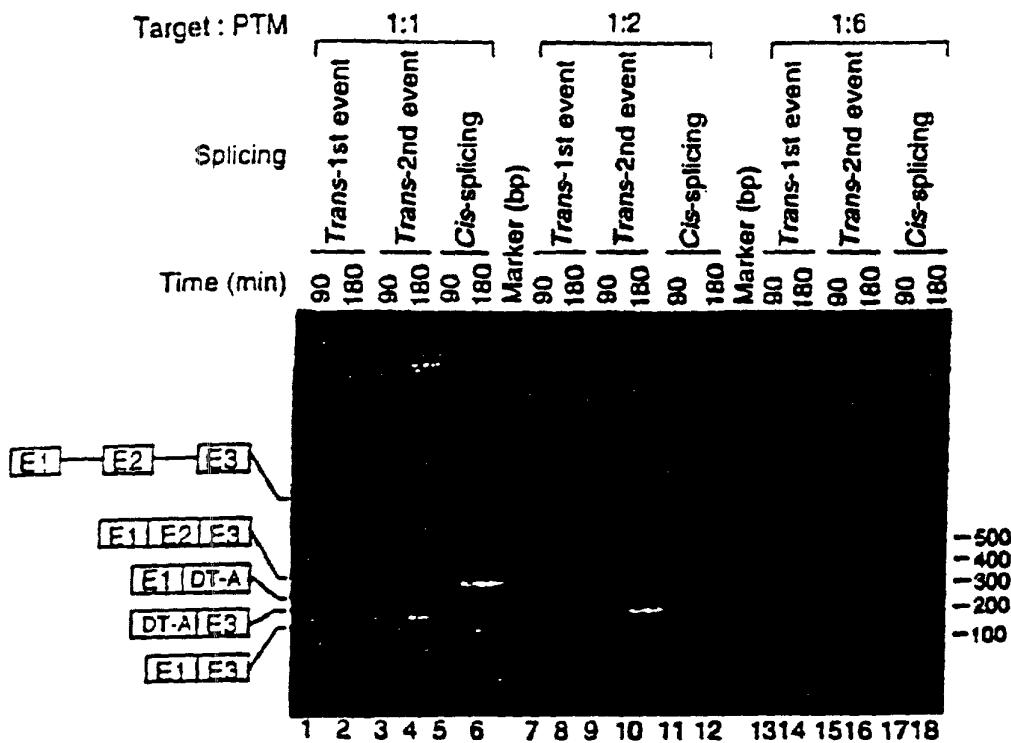
# Double Splicing Pre-therapeutic RNA

Sheet 10 OF 58  
Figure 8A

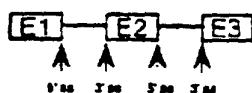


## Selective Trans-splicing of a Double Splicing PTM

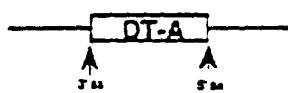
(3' ss of PTM to 5' ss target and, 5' ss of PTM to 3' ss of target)



$\beta$ HCG Target



Double splicing PTM



### Cis-spliced products

**E1|E2|E3** = Normal cis-splicing (277bp)

**E1|E3** = Exon skipping (110bp)

### Trans-spliced products

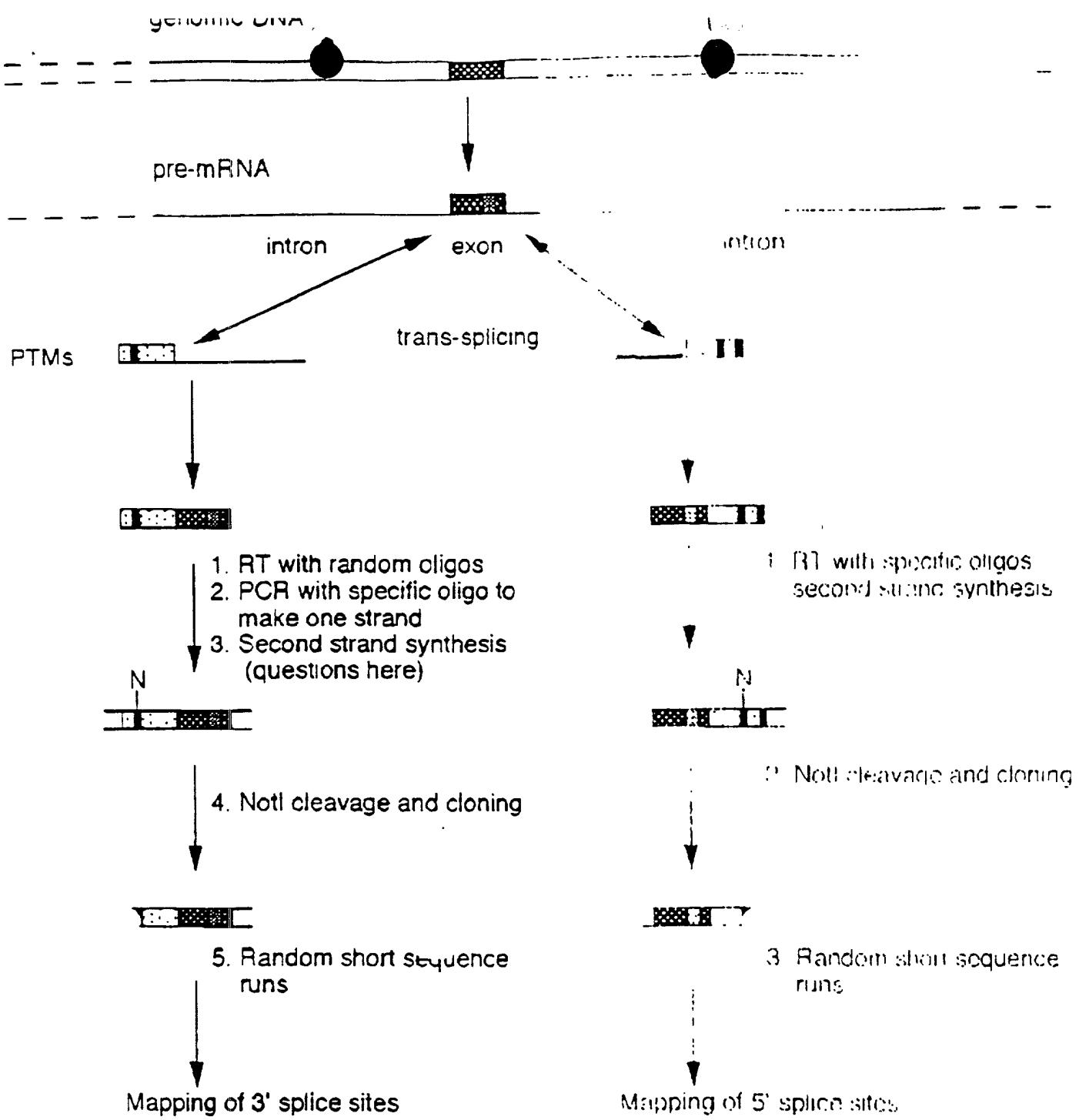
**E1|DT-A** = 1st event, 196bp. Trans-splicing between 5' ss of target & 3' ss of PTM.

**DT-A|E3** = 2nd event, 161bp. Trans-splicing between 3' ss of target & 5' ss of PTM.

Figure 8B

31304B -A

(Sheet 11 Of 58)



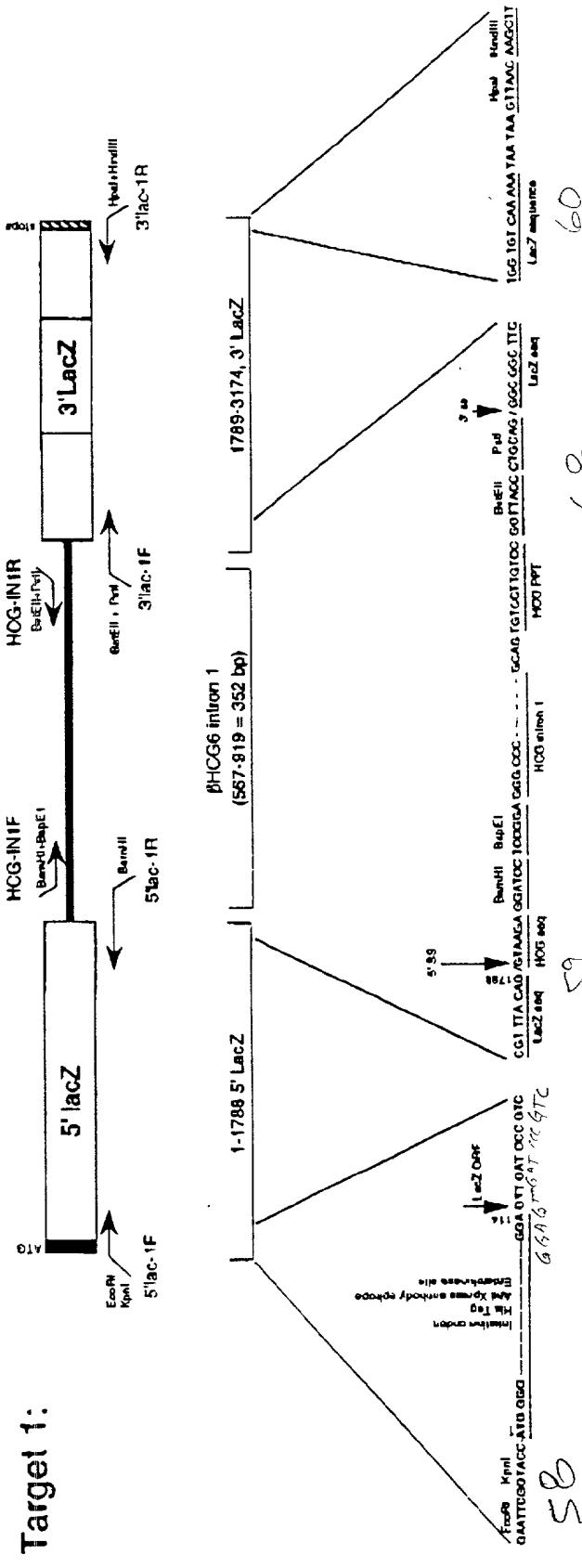
**FIGURE 9**

**31304B-A  
(Sheet 12 Of 58)**

*Knock Out*  
LacZ ~~Marker~~ Model Constructs

$\rho_{\text{C3.1}} \text{ Lac-Z T1}$

Target 1:



58

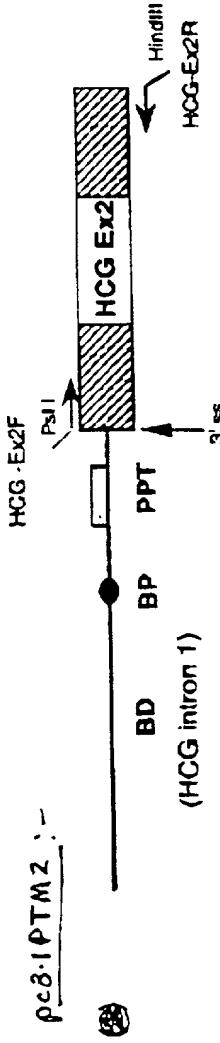
67

59

60

68

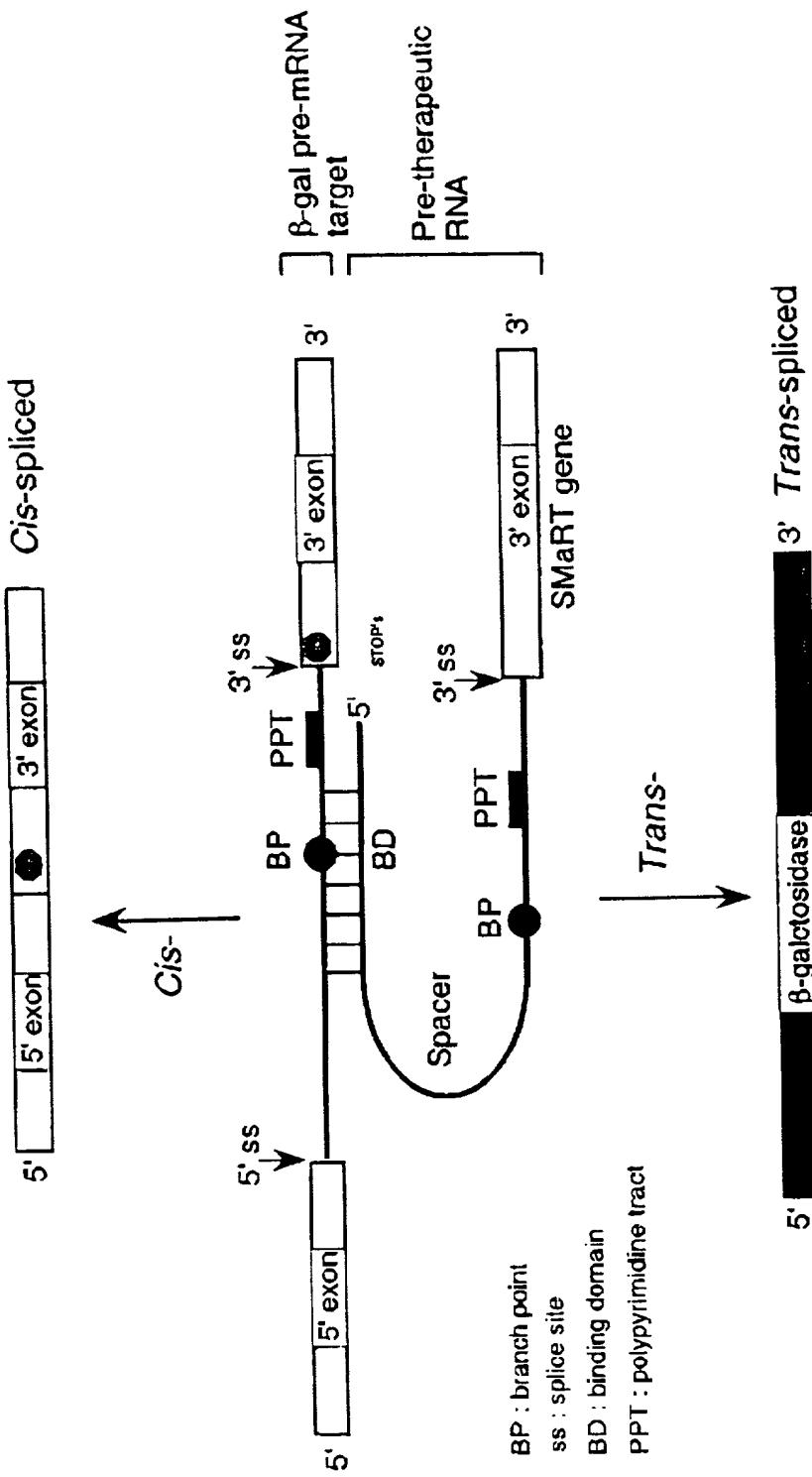
PTMs



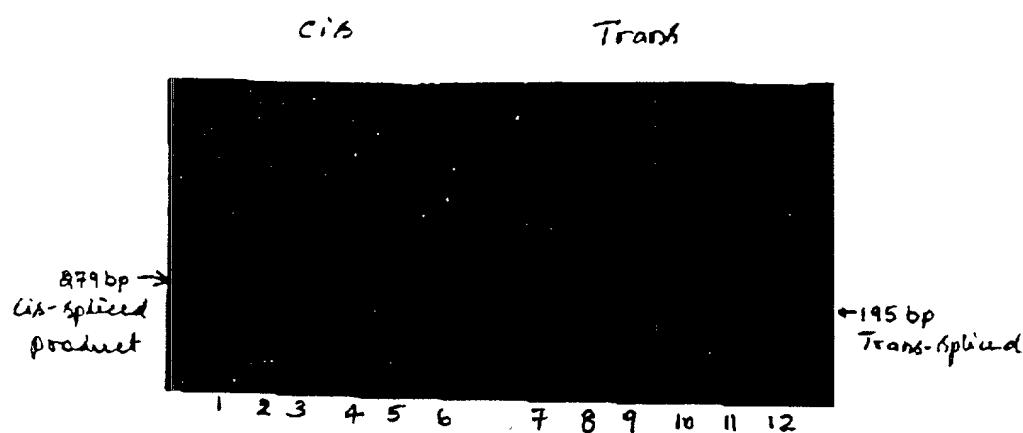
58

31304 B-A  
(sheet 13 of 58)  
FIG. 10 A

# Restoration of $\beta$ -Gal activity by SMaRT (Spliceosome Mediated RNA Trans-splicing)



31304 B-A  
(Sheet 15 of 58)



## FIGURE II A

31304 B-A  
(sheet 16 of 58)

Figure 11B

COOT 10-11

(Shut 17 of 58)

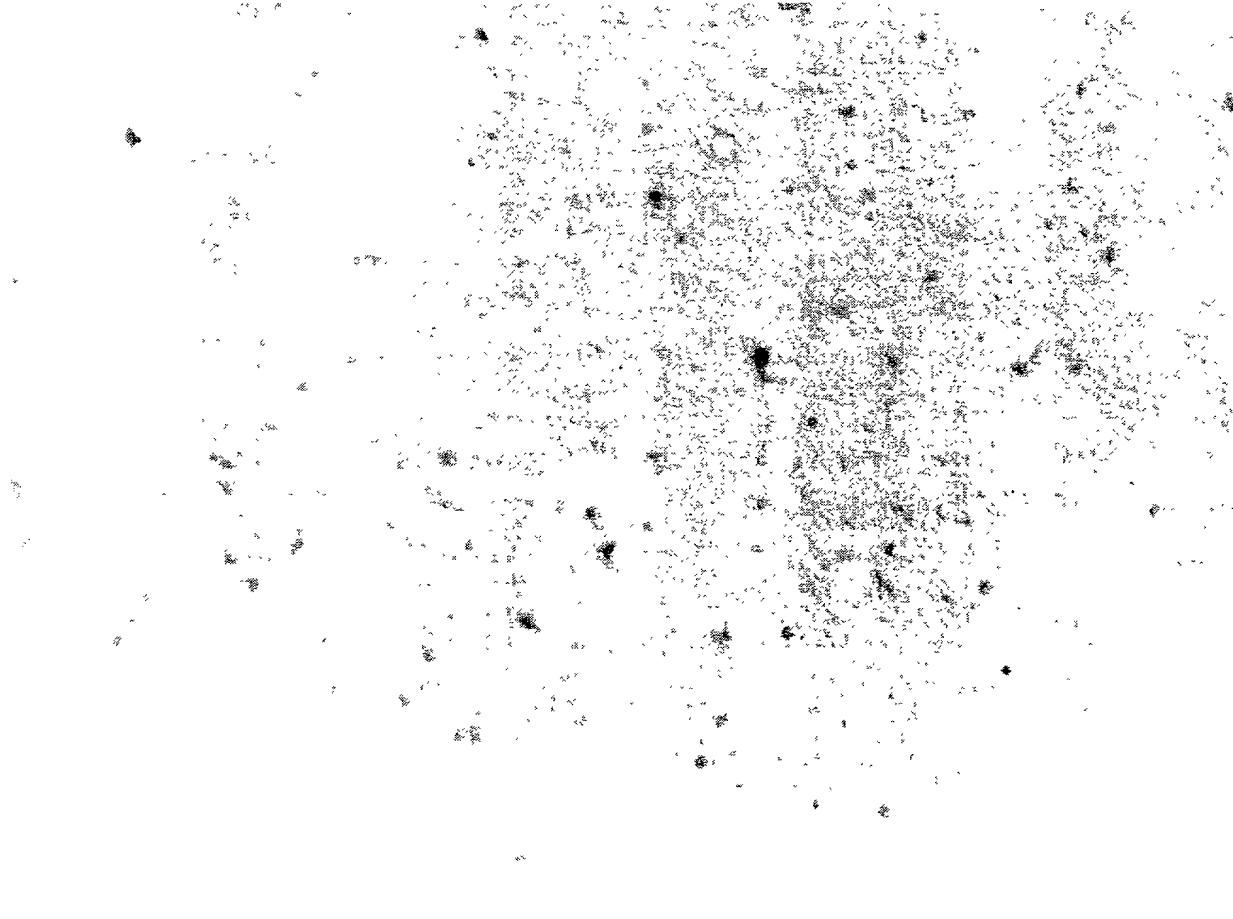


FIGURE 11C

## Nucleotide Sequence Demonstrating that Trans-splicing is Accurate

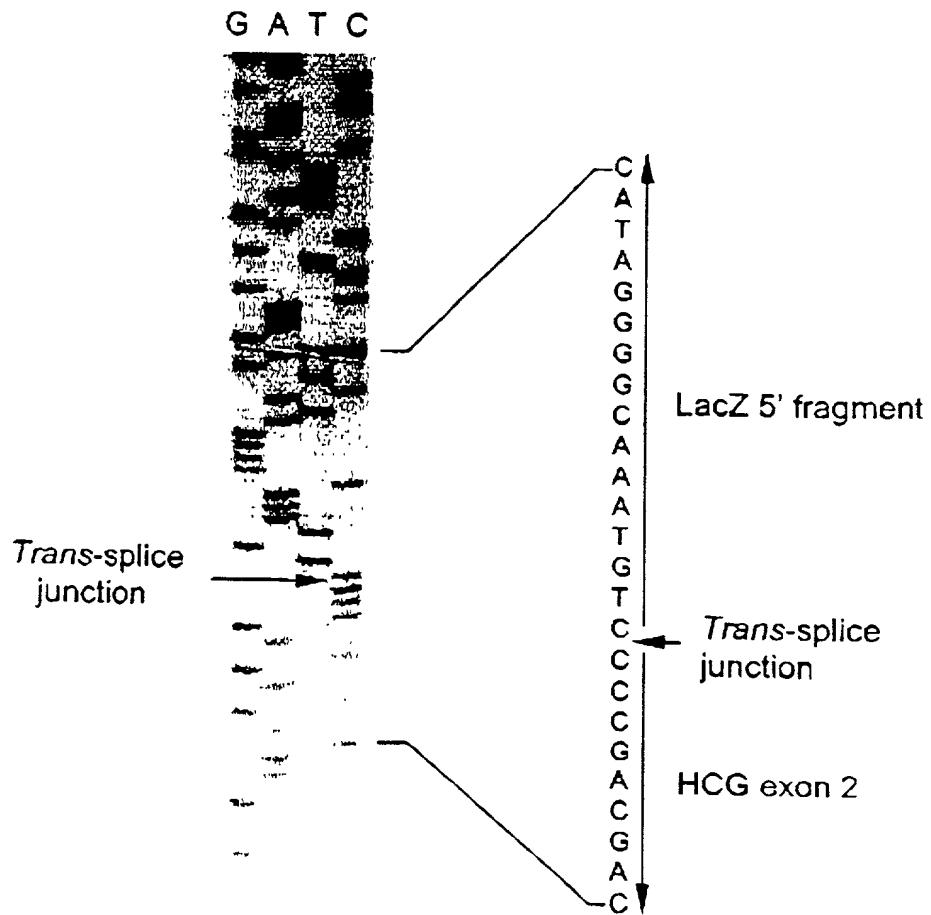


FIGURE 12 A

31304-B-A  
(Sheet 18 of .58)

(1). Nucleotide sequences of the cis-spliced product (285 bp) :

BioLac-TR1

GGCTTTCGCTACCTGGAGAGACGCGCCGCTGATCCTTGCGAATACGCCACGCGATGGTAACAGTCTTG

62

Splice junction

CGGGTTTCGCTAAATACTGGCAGGCAGTTCTGTCAGTATCCCCGTTACAG/GGCGCTTCGTCTAAATAG

GGACTGGTGGATCAGTCGCTGATTAAATATGATGAAAACGGCAACCCGTGGCTGGCTTACGGCGGTGATT

Lac-TR2

TGGCGATAACGCCAACGATGCCAGTTCTGTATGAACGGCTGGCTTGGCGACCCGACGCCATCCAG

(2) Nucleotide sequences of the trans-spliced product (195 bp)

62

BioLac-TR1

GGCTTTCGCTACCTGGAGAGACGCGCCGCTGATCCTTGCGAATACGCCACGCGATGGTAACAGTCTTG

Splice junction

CGGTTTCGCTAAATACTGGCAGGCAGTTCTGTCAGTATCCCCGTTACAG/GGCTGCTCTCTCTGCTGCT

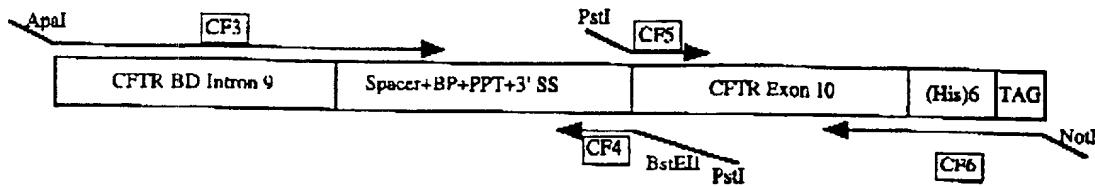
HCR2

GAGCATGGCGGGACATGGCATCCAGGAGCCACTTCGGCACGGTGCCG

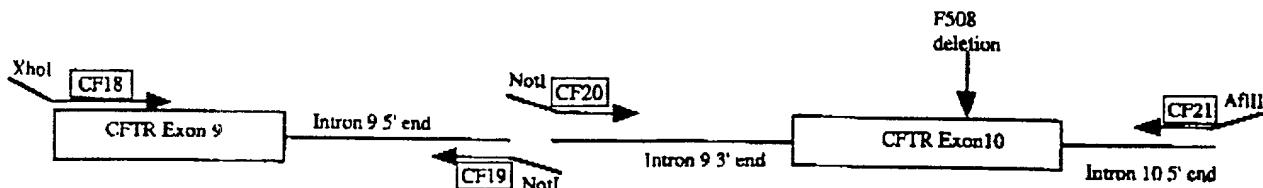
Figure 12 B

31304 - B-A  
(Sheet 19 of 58)

## CFTR Pre-therapeutic molecule (PTM or "bullet")

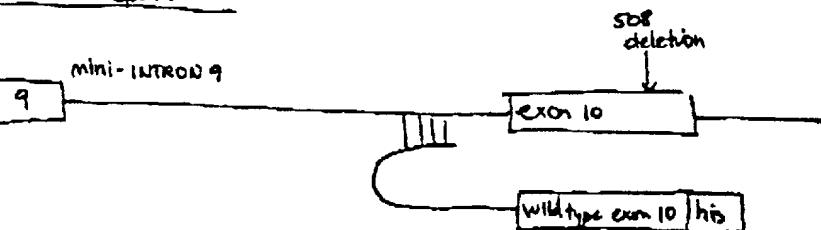


## CFTR mini-gene target - Construction



### TRANS- SPLICING Repair

Binding  
of  
PTM to TARGET



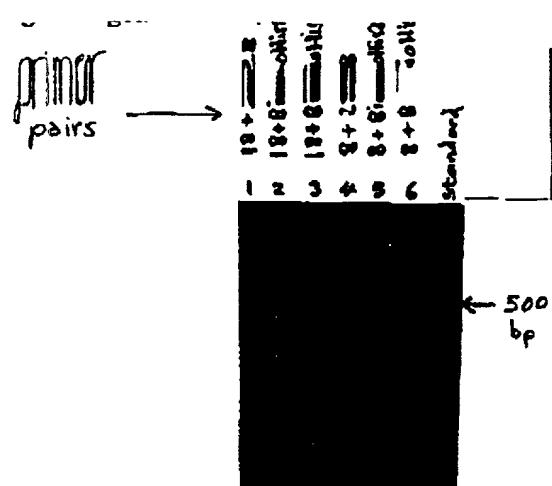
↓ splicing

exon 9      wild type exon 10 ) his

Figure 13

31304-B-A  
(sheet 20 of 58)

Figure 14

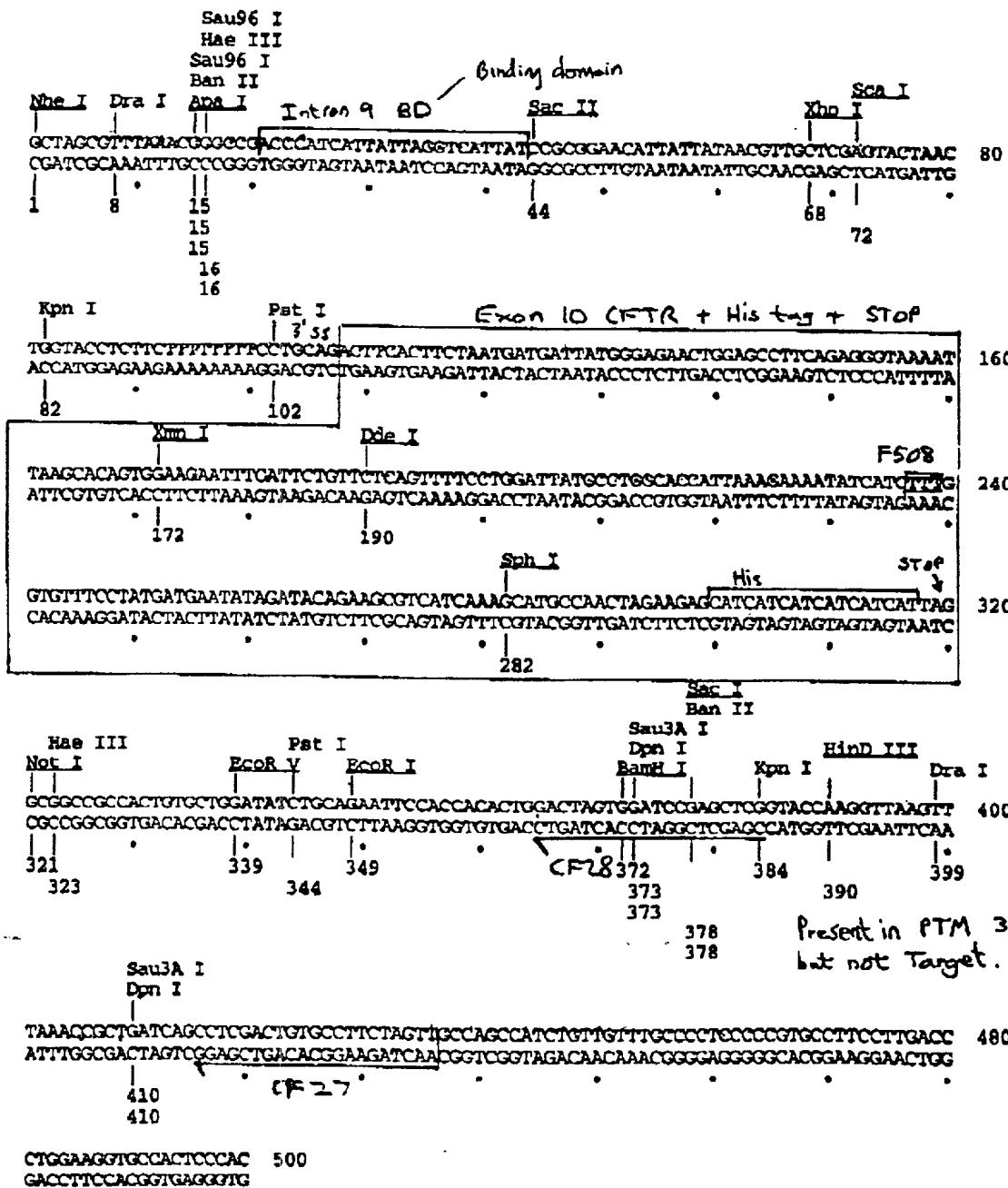


31304 B-A  
(Shut 21 of 58)

# FIGURE 15

DNA sequence 500 b.p. GCTAGCGTTAA ... TGCCACTCCAC linear /

Positions of Restriction Endonucleases sites (unique sites underlined)



## Restriction Endonucleases site usage

Acc I	-	EcoR I	1	Nde I	-	Sau96 I	2
Apa I	1	EcoR V	1	Nhe I	1	Sca I	1
Apal I	-	Hae II	-	Not I	1	Sma I	-
Avr II	-	Hae III	2	PflM I	-	Sph I	1
BamH I	1	HinC II	-	Pst I	2	Spl I	-
Ban II	2	HinD III	1	Pvu I	-	Ssp I	-
Bbs I	-	Hinf I	-	Pvu II	-	Stu I	-

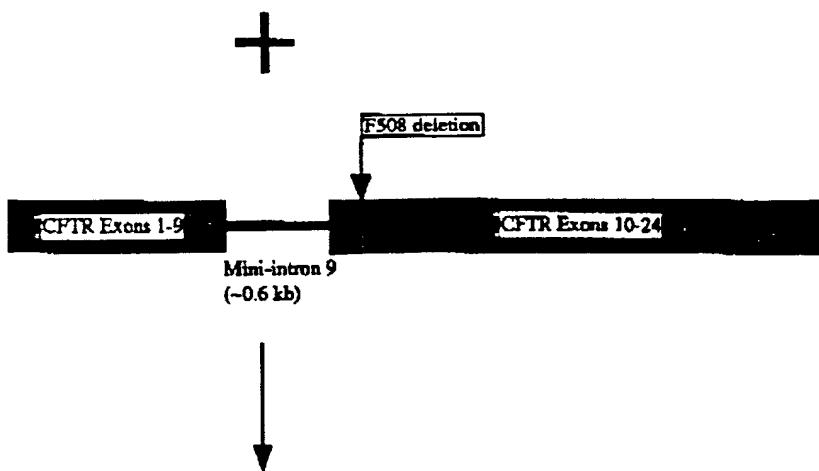
31304-A-B  
(Shut. 22 of 58)

**EXPERIMENT 2**

Repair of an exogenously supplied CFTR target molecule carrying an F508 deletion in exon 10.

PTM

CFTR Target  
(mini-gene)



Cotransfect PTM and Target molecules in HEK 293 cells  
and detect repaired CFTR mRNA by RT-PCR.

Repaired  
CFTR mRNA



Figure 1b  
31304-A-B  
sheet 23 of 58

FROM Intronn 9196862129

**EXPERIMENT 3**

Repair of endogenous CFTR  
transcripts by exon 10 invasion  
using a double splicing PTM

**Double Splicing  
PTM**

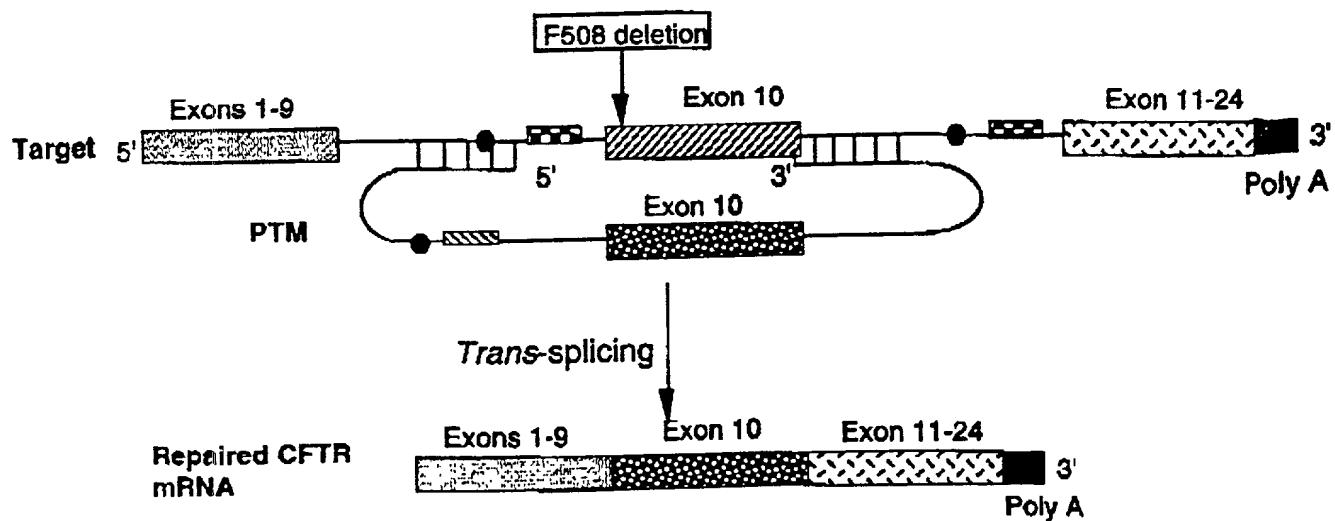
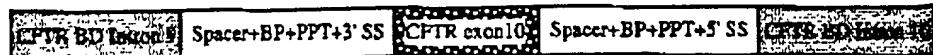


Figure 17

31304 B-A

Sheet 24 of 58

## Double Trans-splicing Specific Target

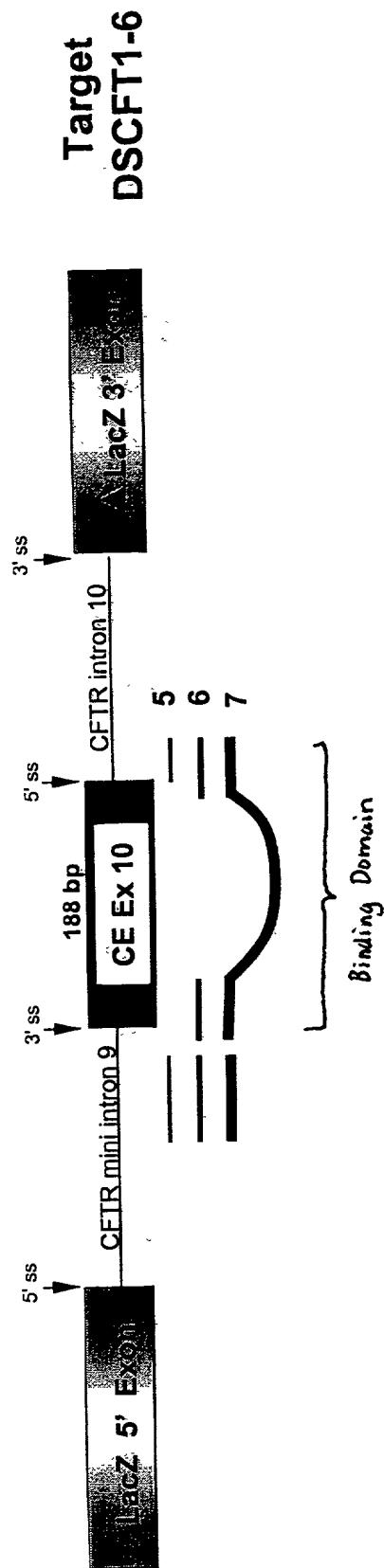


Figure 18

About 25 of 58

# Double Trans-splicing PTMs

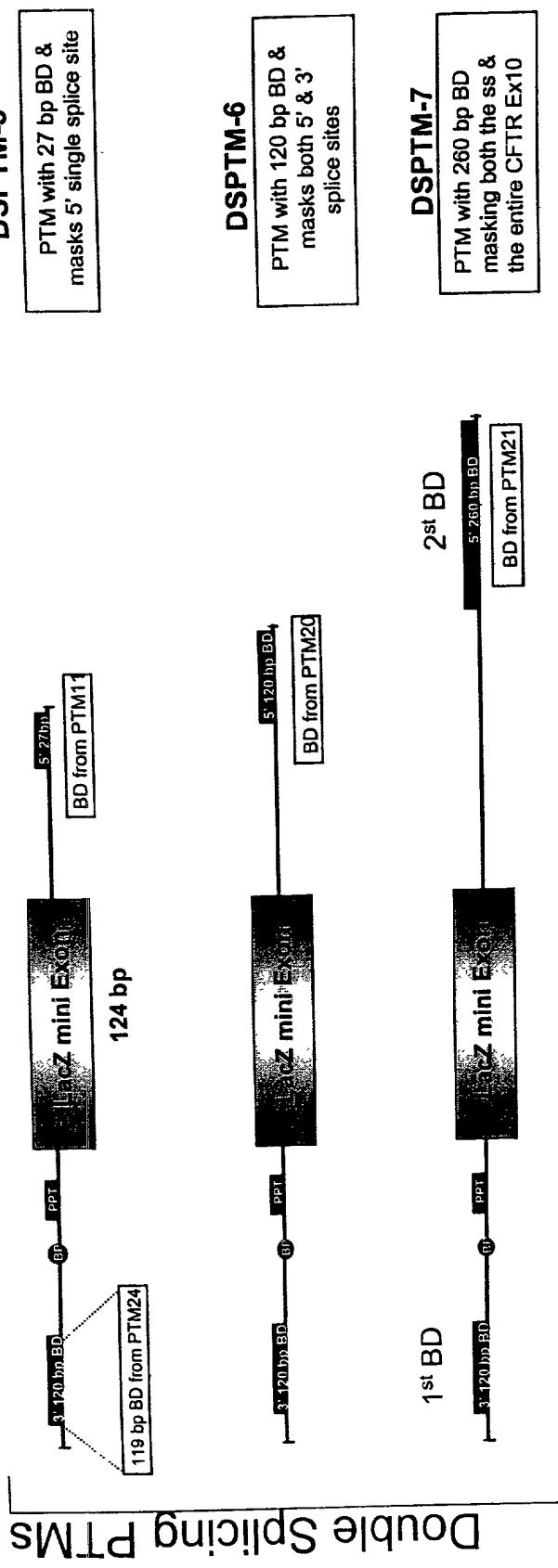


Figure 19

about 26 of 58

## Double Trans-splicing $\beta$ -Gal Model

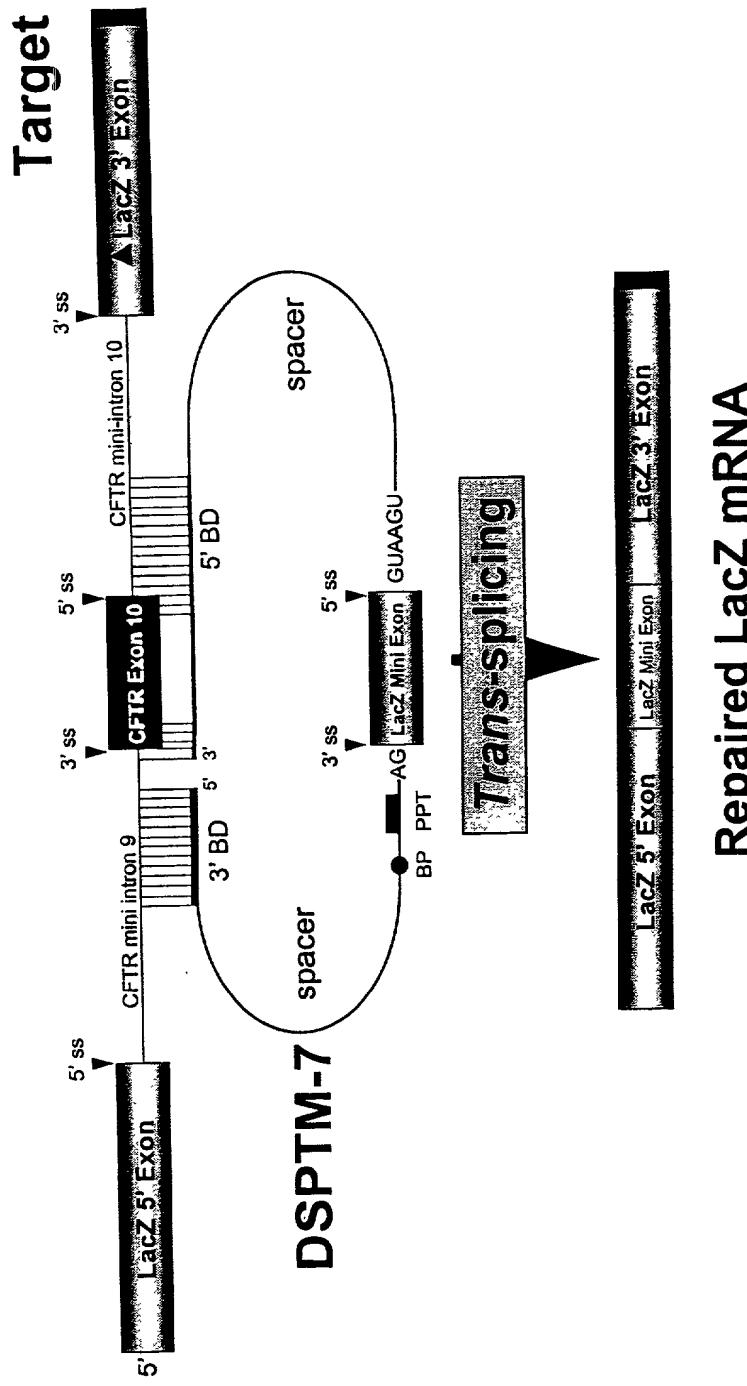
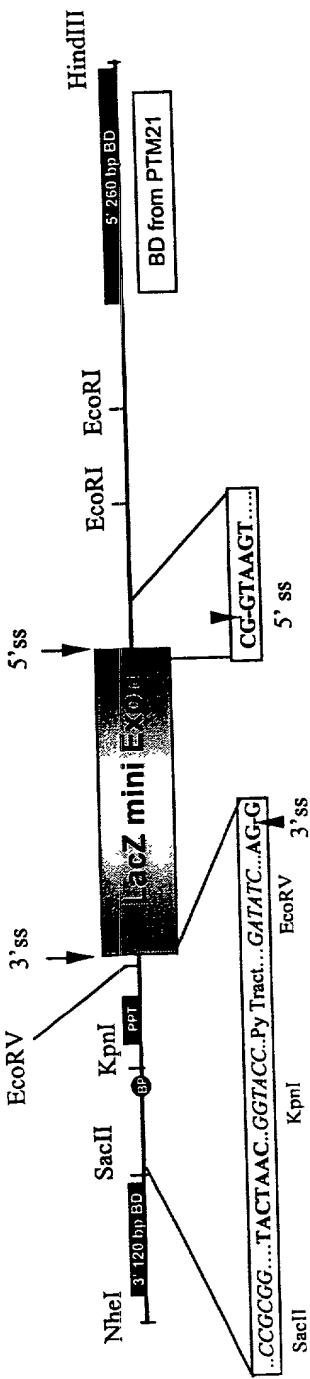


Figure 20

85 to 78

**Important Structural Elements of DSPTM-7:** (Double splicing PTM with all the necessary splice elements i.e. has both 3' and 5' functional splice sites and the binding domains)



(1) 3' BD (120 BP) : GATTCACTTGTCCCAATTATCATCCTAAGCAGAAGTGTATACTTCTTATTGTAAAGATTCTATTAAACTCATTGATTCA  
AAAATATTAAATAACCTTCCTTTCATACTCTGCTATGCAC

(2) Spacer sequences (24 bp): AACATTATTAAACGTTGCTCGAA

(3) Branch point, pyrimidine tract and acceptor splice site: TACTAAC T GGTACC TCTTCTTTTTT GATATC CTGCAG

(4) 5' donor site and 2<sup>nd</sup> spacer sequence:  

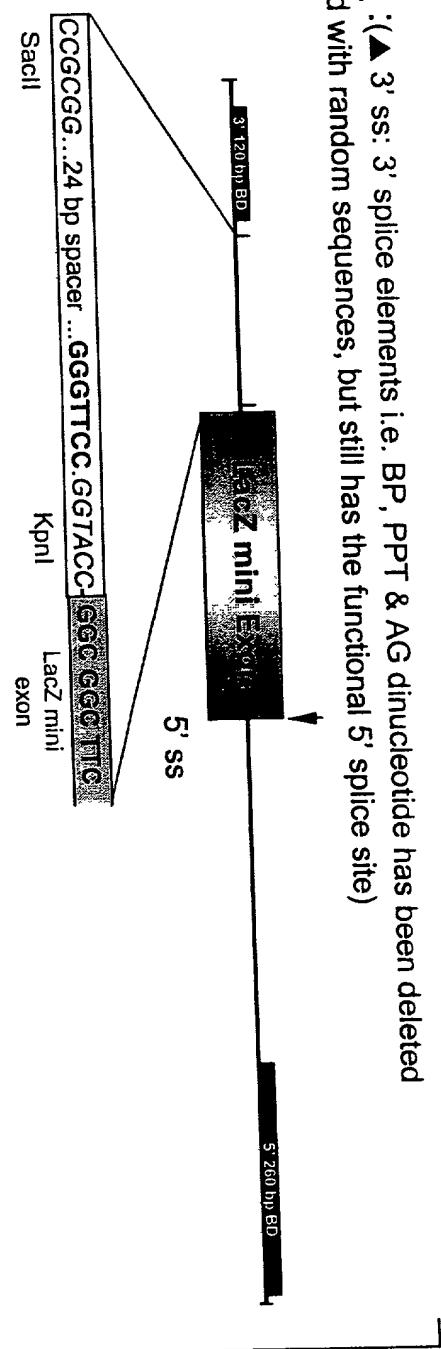
BP      Kpn I      PPT      EcoRV  
 LacZ mini exon      5' ss      LacZ mini exon

The sequence starts with a LacZ mini exon (TCAAGATCCACGGG), followed by a 5' ss (TCAAGTGTAAAGTGTAAATCTTACCTCTTCAATAATTCTTACCTCTTCAATTGCTGGCTGGATAATCCTGAAATTCTCCATTCTGAATTCTCCATTCTGAACTCTGGAAATAAAACCCATCATTACAACCTGAACTCTGGAAATAAAACCCATCATTATAACTCA

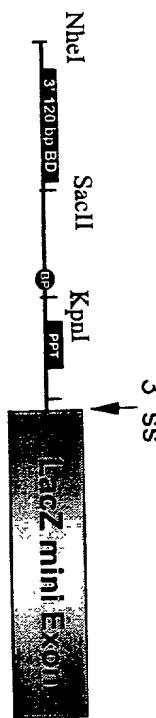
(5) 5' BD (260 BP) : TCAAAAAGTTTCAATAATTCTTACCTCTTCAATTGCTTGTAAATTCAATGCTTGTGCTTGTGCTTAAACACAAATGAAATTCTTCCACTGTGCTTAAACACAAATGATAAATGGAAATTCTTCCATTCTGAATTCTCCATTCTGAATTCTCCATTCTGAACTCTGGAAATAAAACCCATCATTATAACTCA

Figure 21

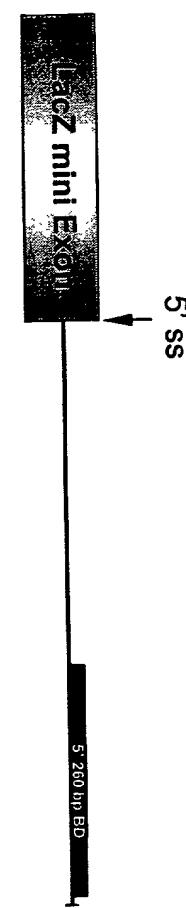
**DSPTM8** : (▲ 3' ss: 3' splice elements i.e. BP, PPT & AG dinucleotide has been deleted and replaced with random sequences, but still has the functional 5' splice site)



**PTM29** (lacks 2<sup>nd</sup> BD and 3' ss)



**PTM30** (lacks 1<sup>st</sup> BD and 3' ss)



Mutants

Figure 22

## Accuracy of Double Trans-splicing Reaction

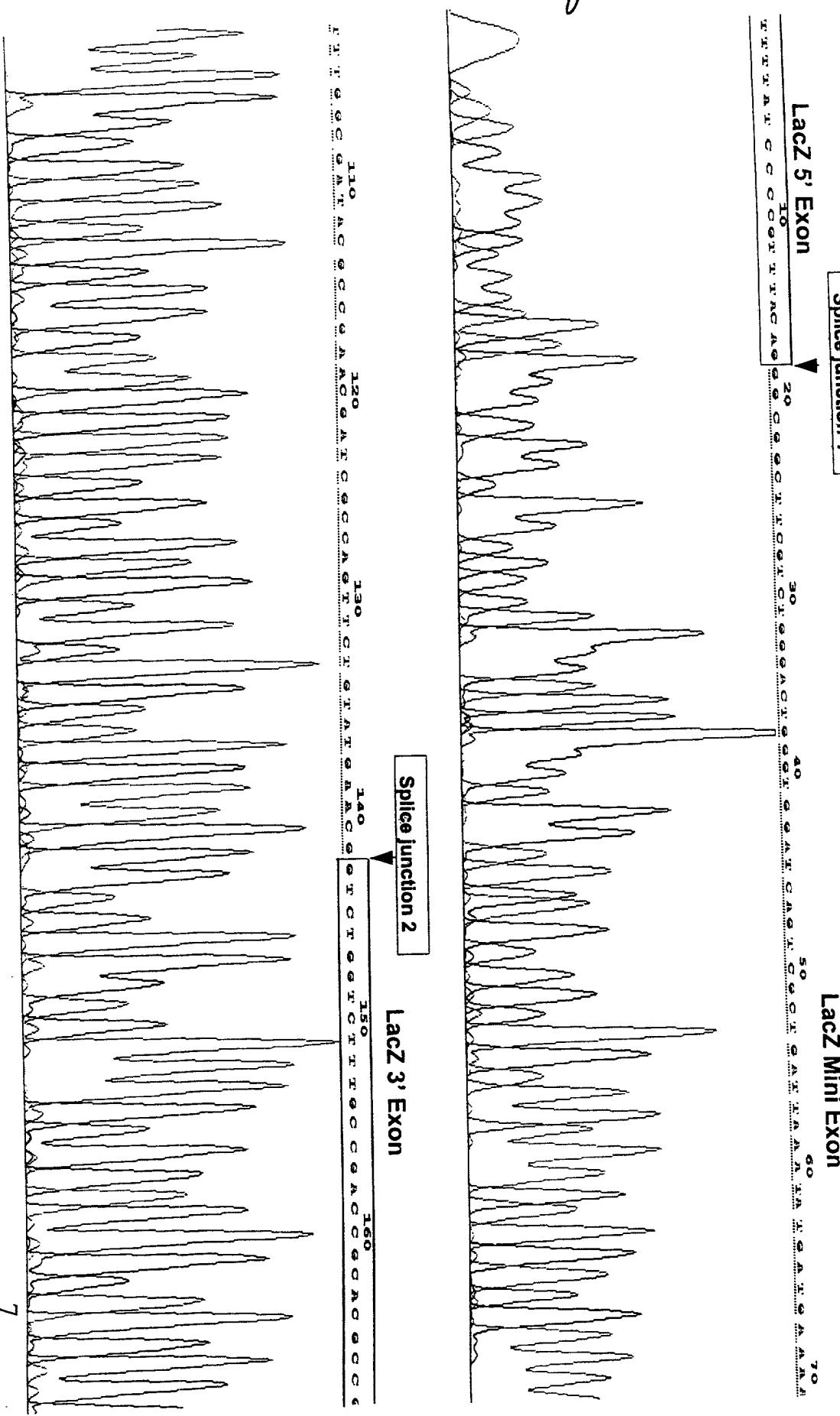
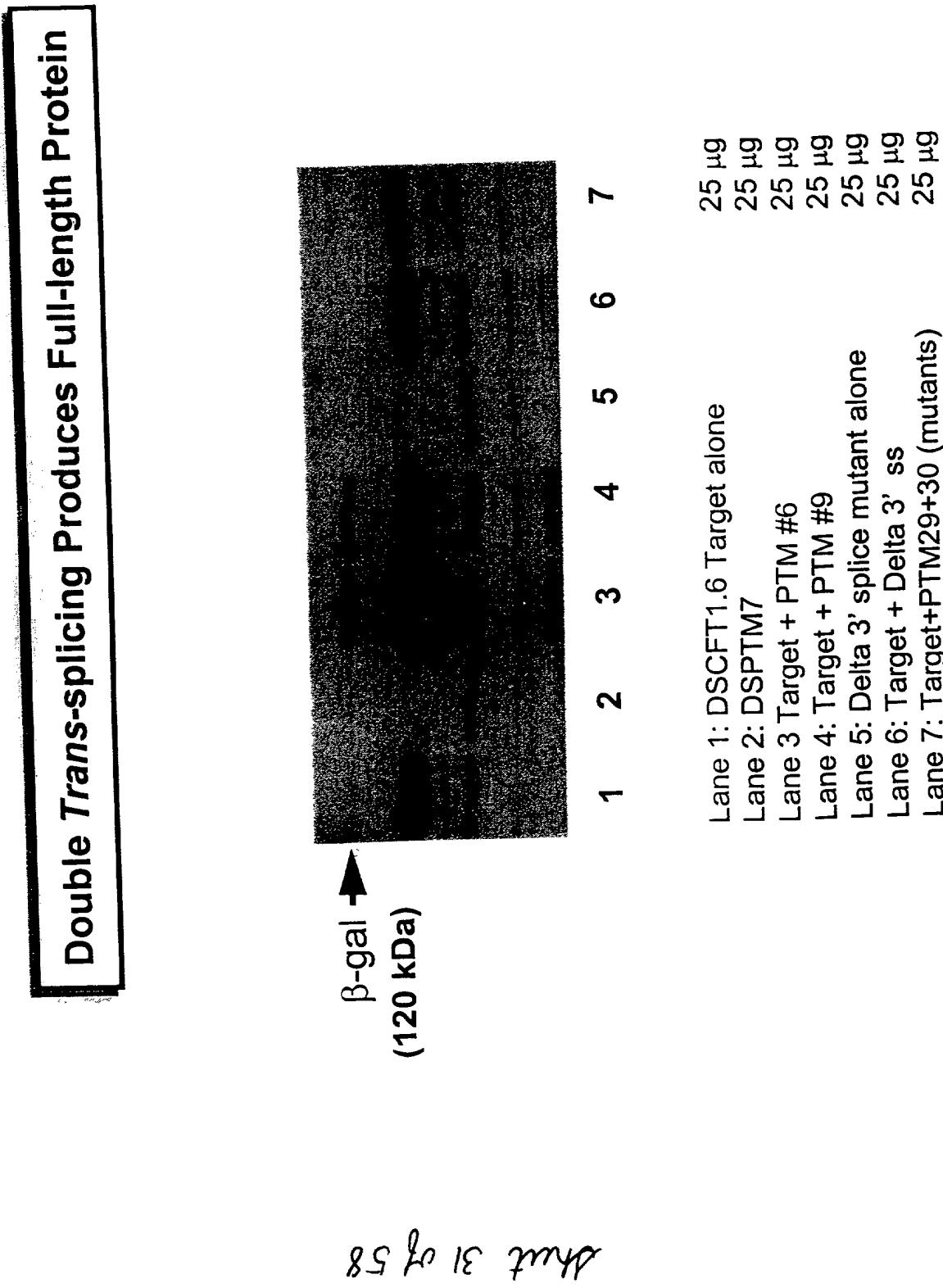
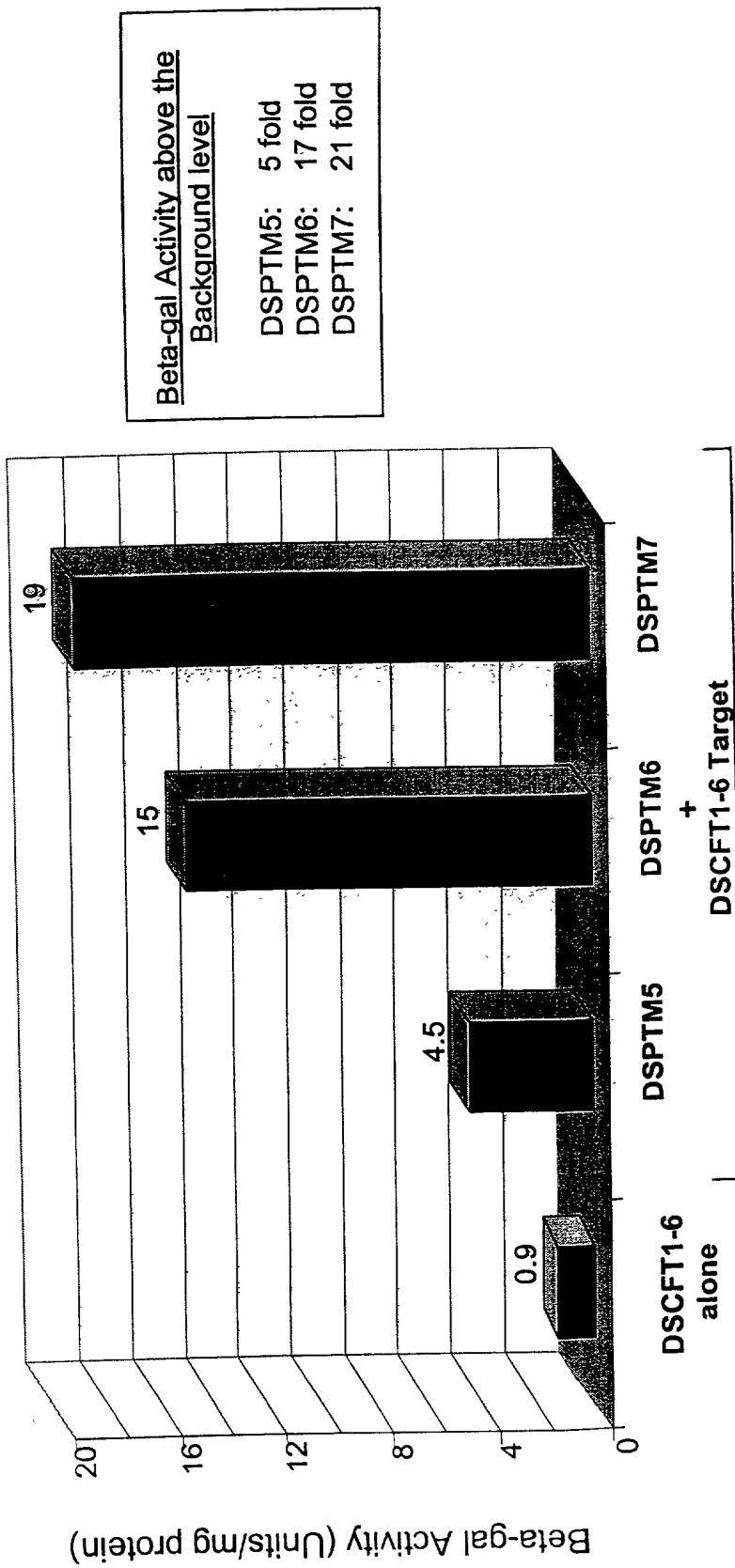


Figure 23

Figure 24



## Restoration of $\beta$ -Gal Function by Double Trans-splicing



What 32 of 58

## Restoration of $\beta$ -gal activity is due to double RNA trans-splicing events

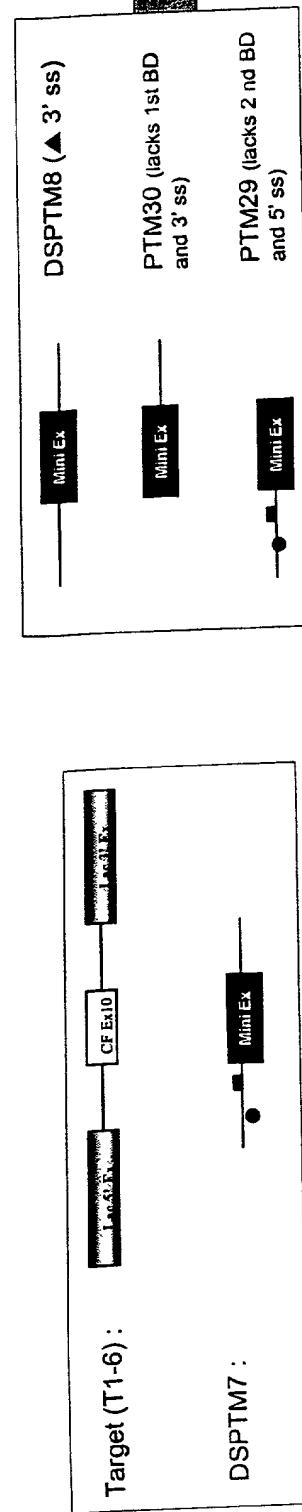
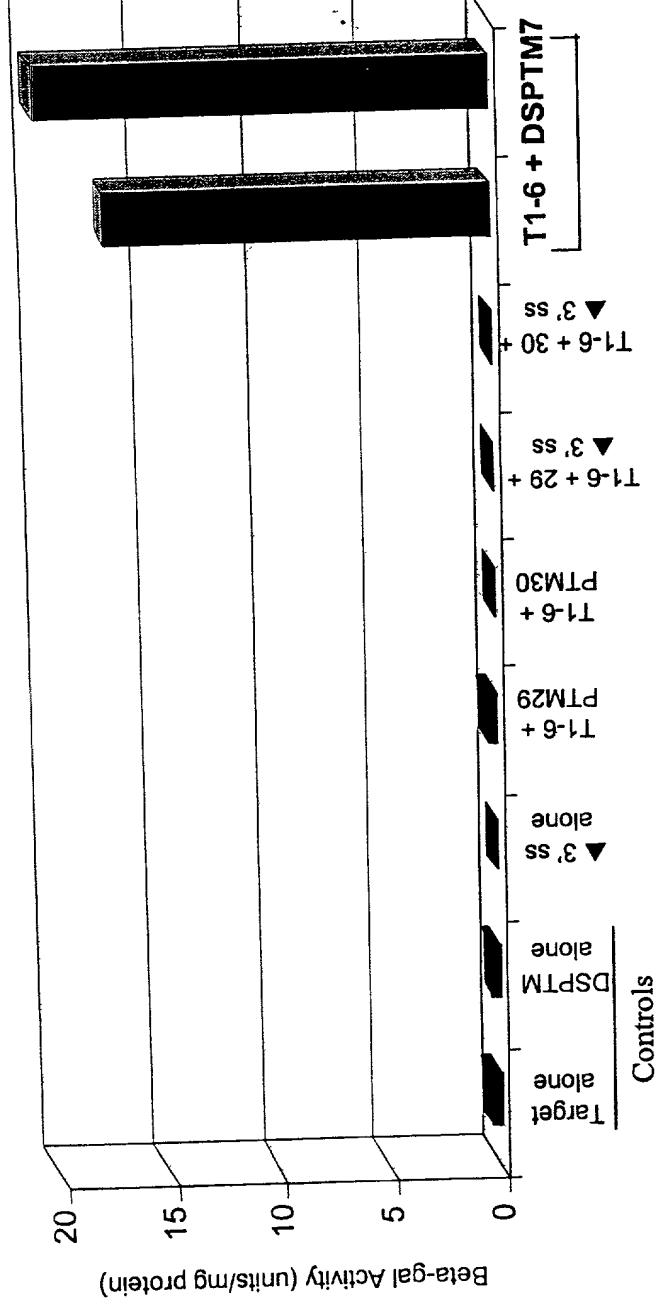


Figure 26

8 of 33 dhw

## Double Trans-splicing: Titration of Target & PTM

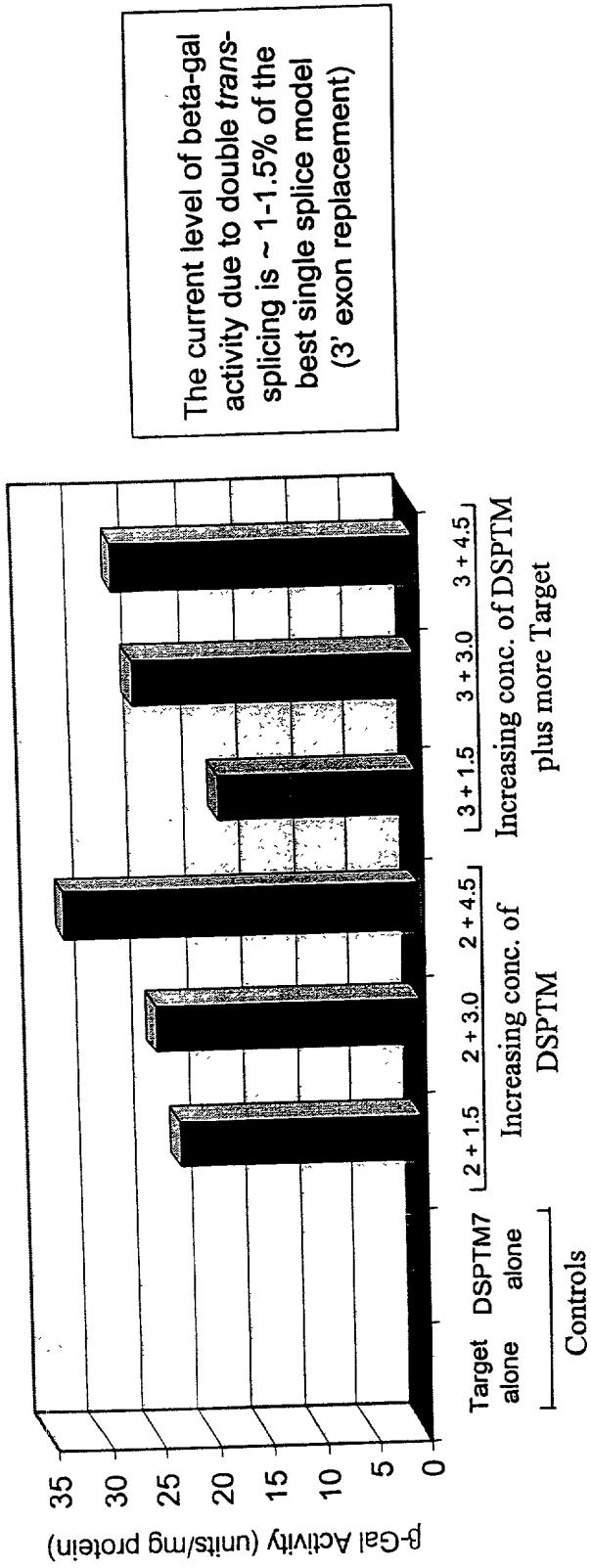
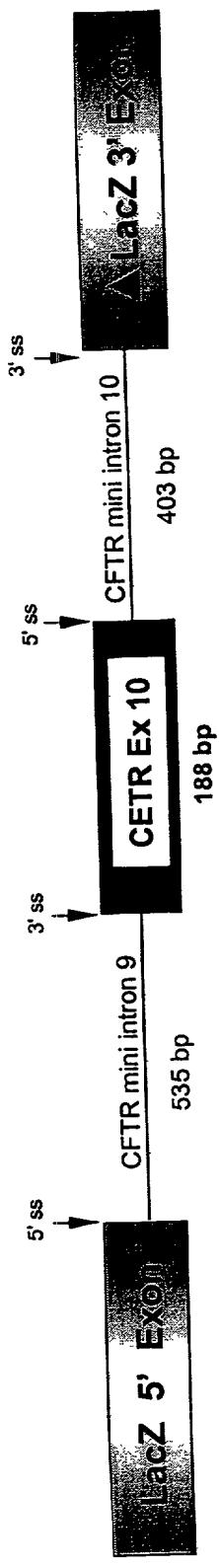


Figure 27

Chart 34 of 58

### DSCFT1-6 (Specific Target):



### DSHCGT1 (Non-specific Target):

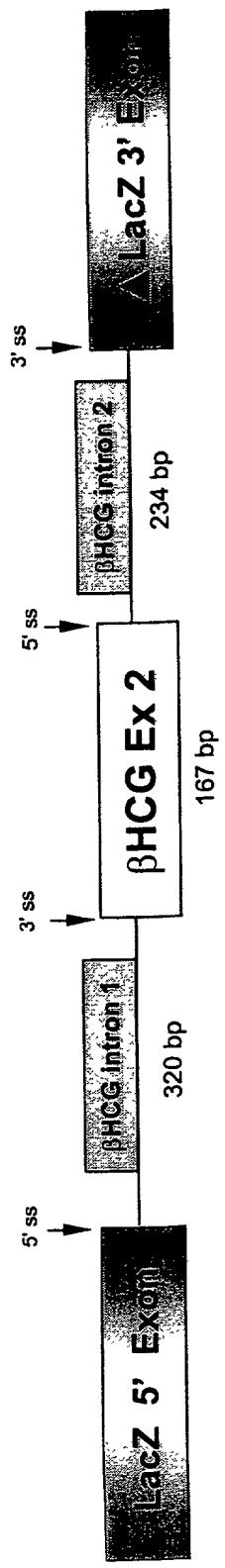


Figure 28

that 35 of 58

## Specificity of double *trans*-splicing Reaction

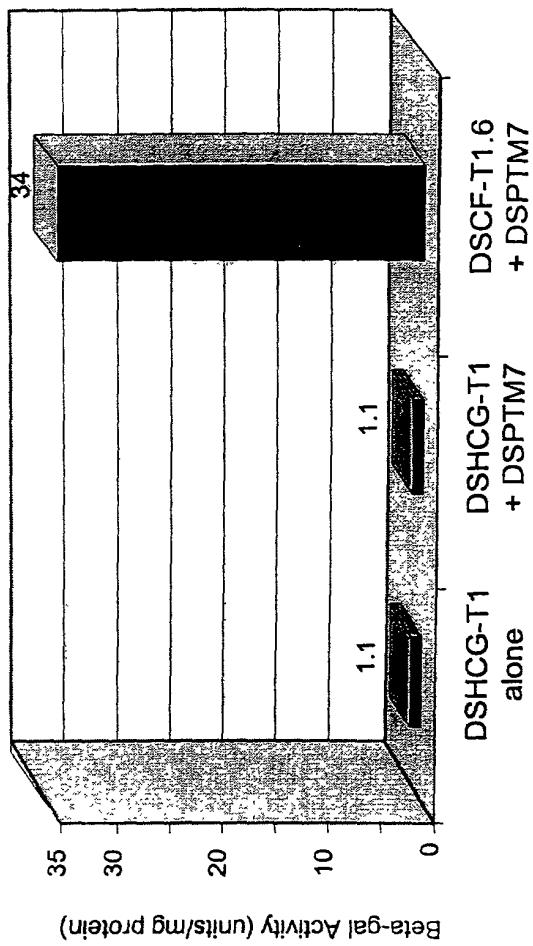
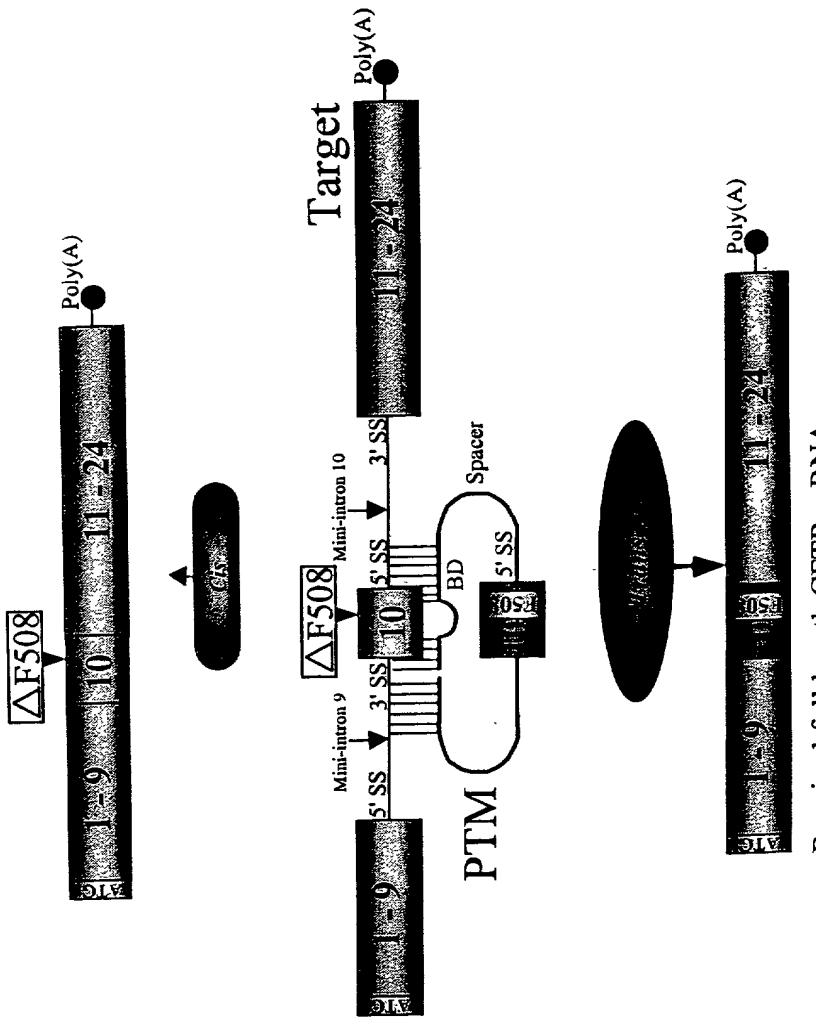


Figure 29

about 36 of 58

卷之三



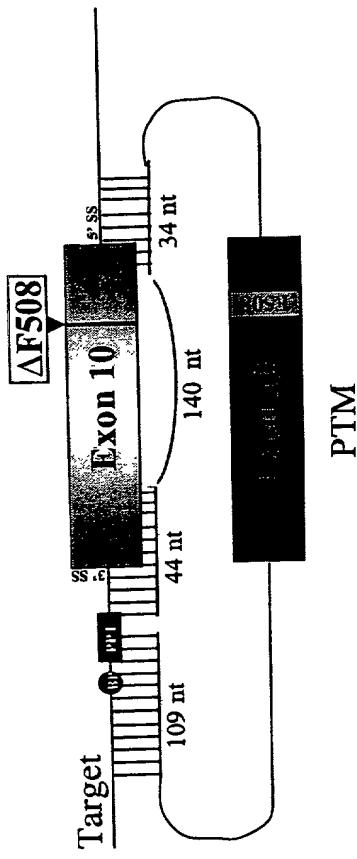
### Repaired full length CFTR mRNA

ENVIRONN

Figure 30

Target gene contains two splice sites and part of exon 10 in a mini-gene target.

PTM with a long binding domain masking  
two splice sites and part of exon 10  
in a mini-gene target.



ACGAGCTTGCCTCATGATGATCATGGGCCAGTTAGAACCAAGTGAAGGCCAA**GATC**AAACAT**TCCG**

GCCCCATCAGCTTTCGCAGCCAA**ATT****CAGT**GGATCATGCC**GGT**ACCAT**CAAG****GAA****CATAAA**

**T****T****T****GGCCGT****CAGTTACGAGTACCG****GATTAAGG****CTGCTCGGGT****GATT****TGGA****GGAG**

#### MCU in exon 10 of PTM

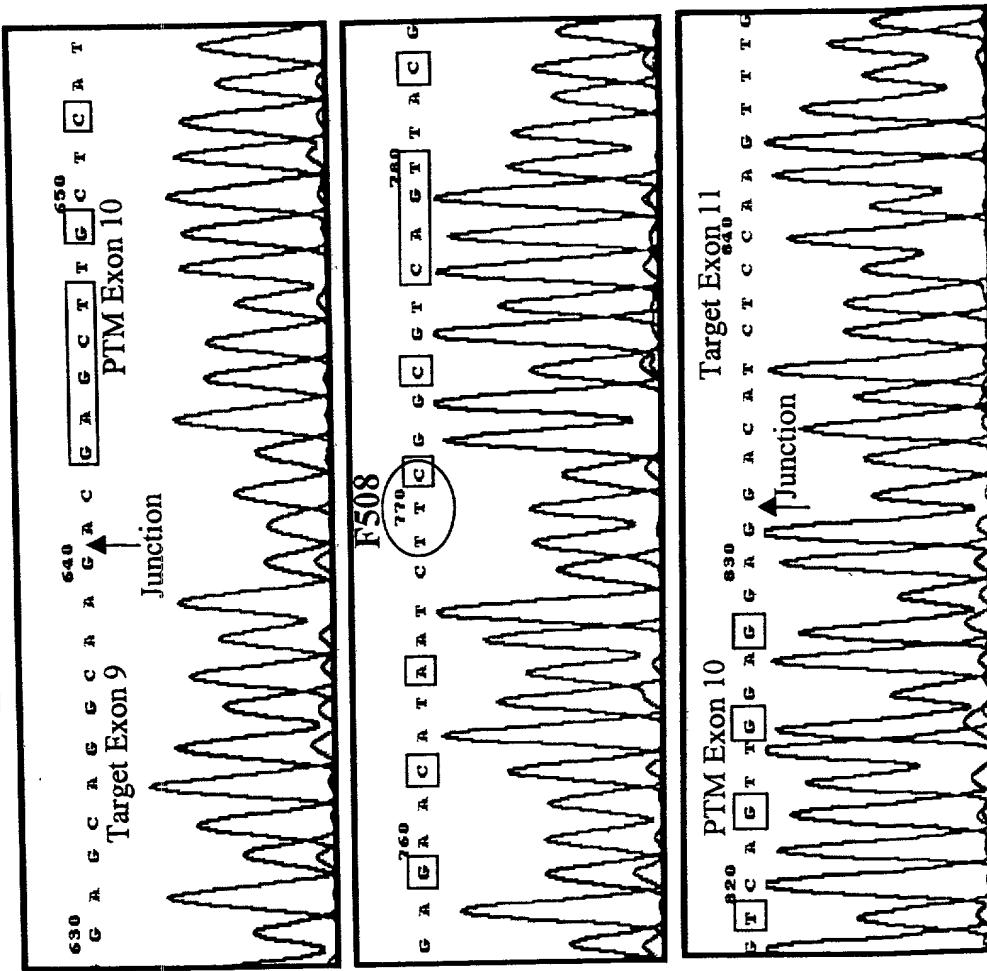
88 of 192 (46%) bases in PTM exon 10 are not complementary to its binding domain (bold and underlined).

Figure 31

INTRODUCTION

85 to 88 myd

Sequence of a double  
trans-spliced product



= MCU in  
PTM exon 10

Figure 32

85 to 39 mut

**CETTR Repair: 5' Exon Replacement**  
**Schematic diagram of a PTM binding to the splice site of**  
**intron 10 of a mini-gene target**

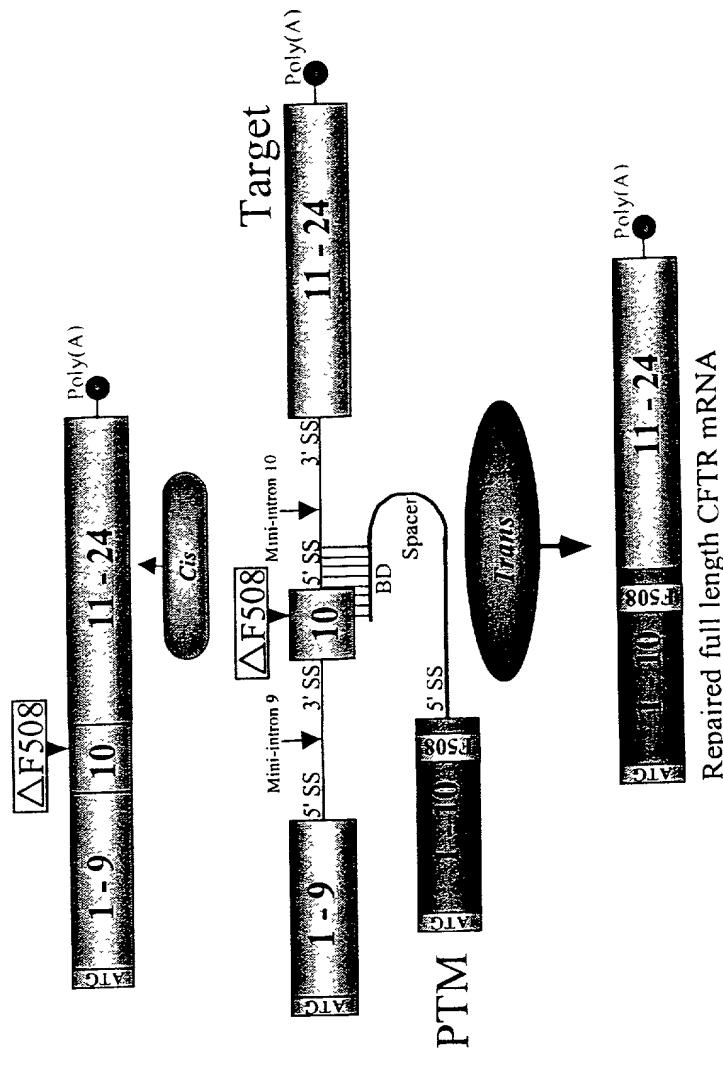
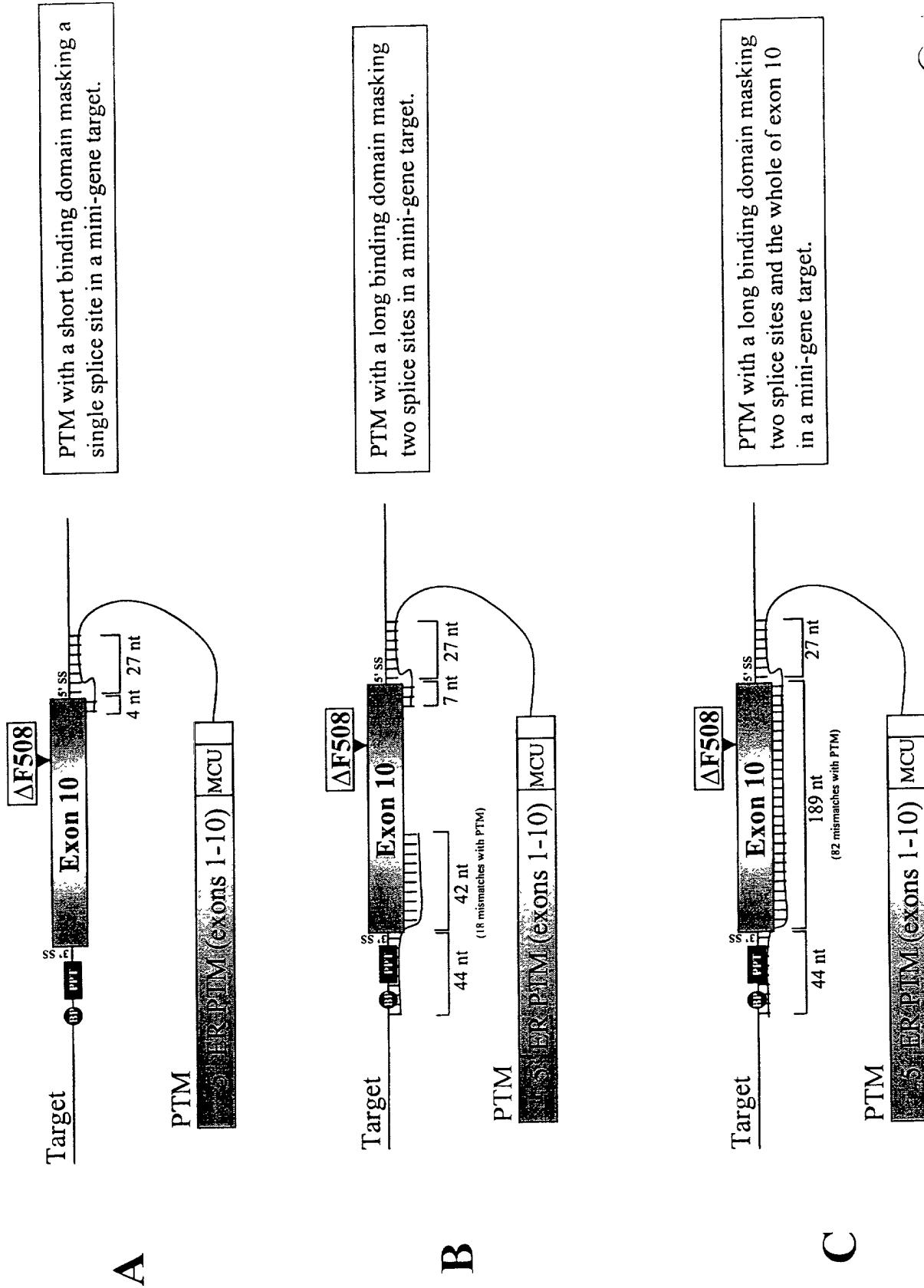


Figure 33  
 Repaired full length CFTR mRNA

Figure 34



85 to 147 myr

Sheet 42 of 58

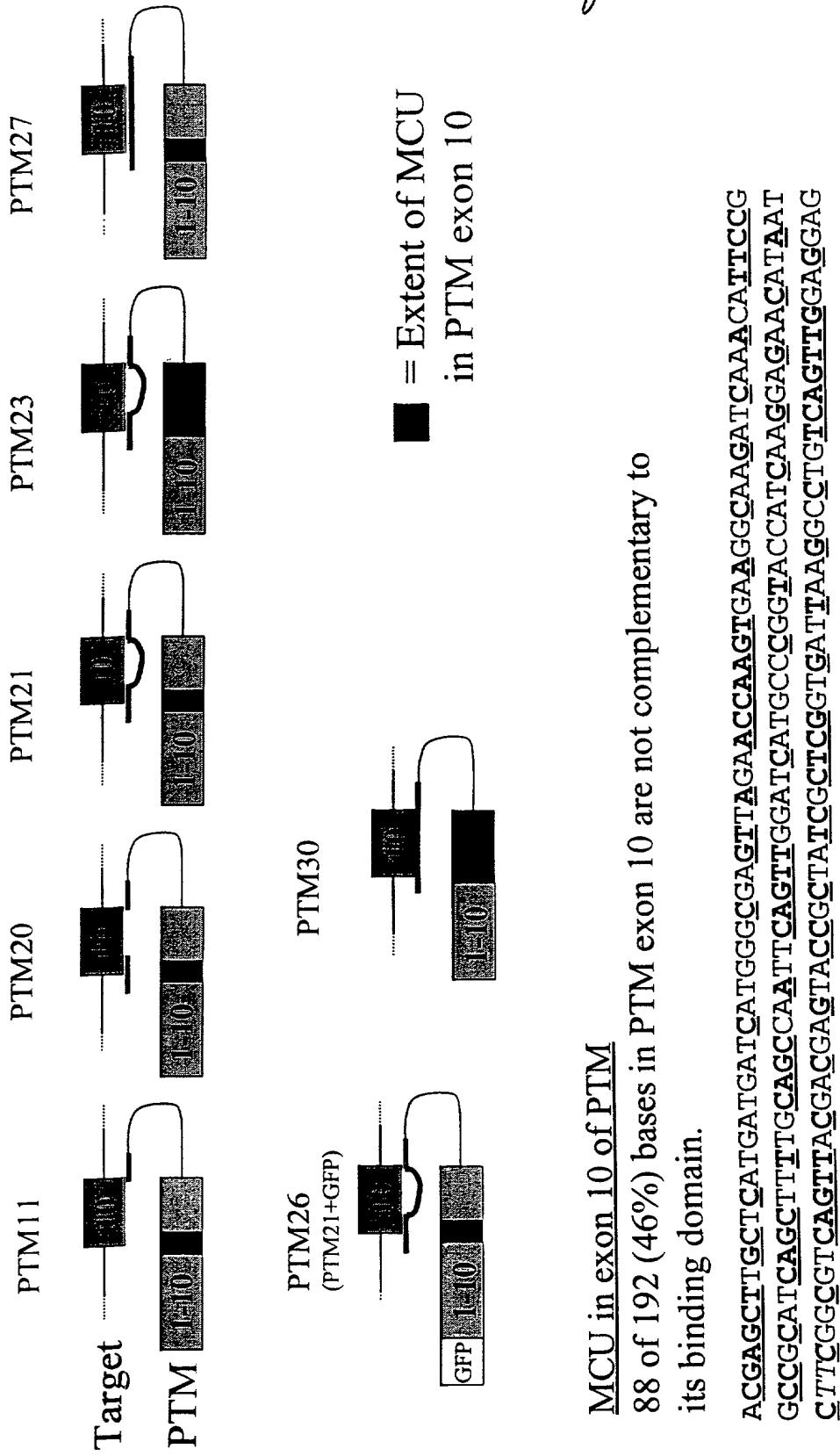
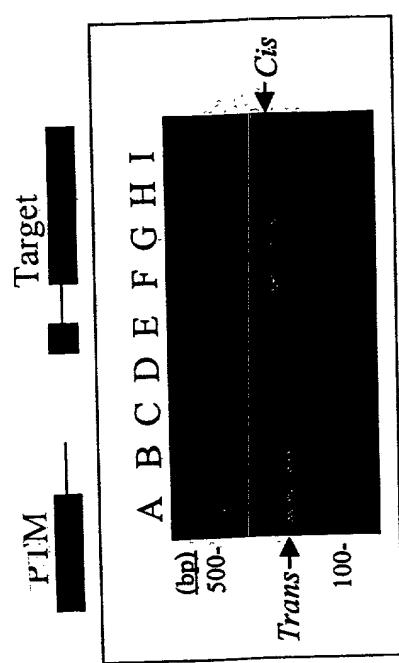
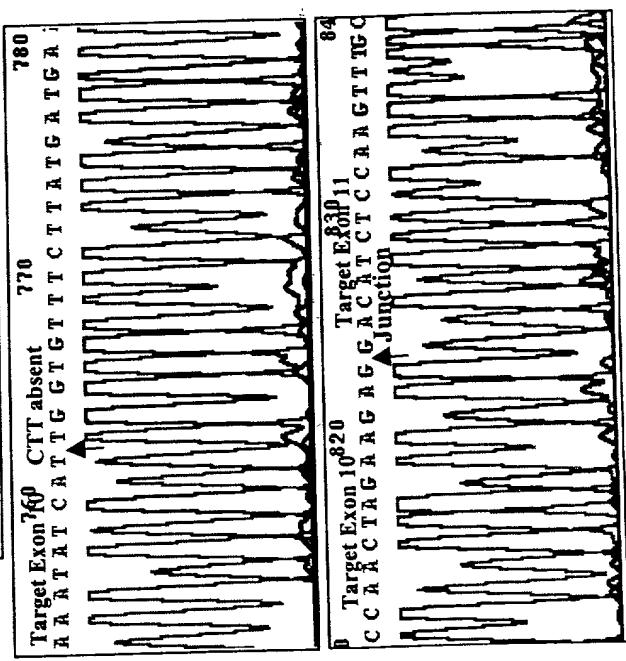


Figure 35

INTRON



**A.** Cis-Spliced product  
[Primers CF1 + CF111]



**B.** Trans-spliced product  
[Primers CF93 + CF111]

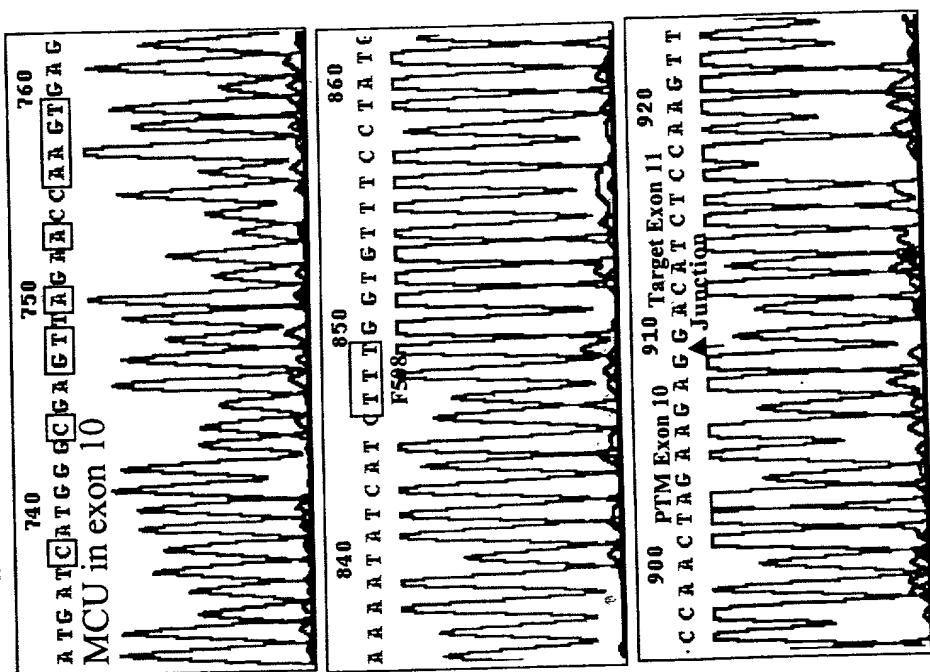
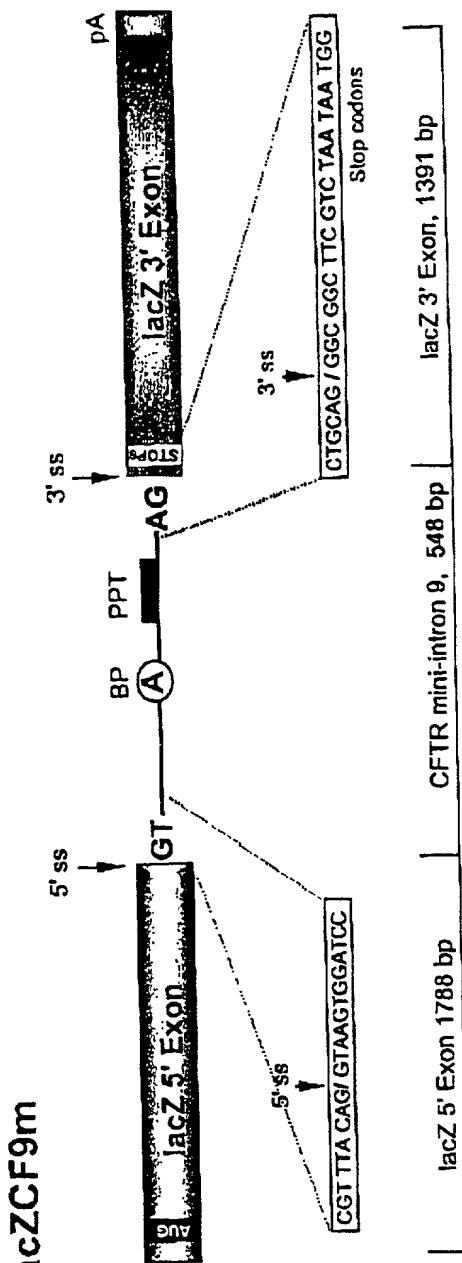


Figure 36

as to Et myr

Sheet 44 of 58

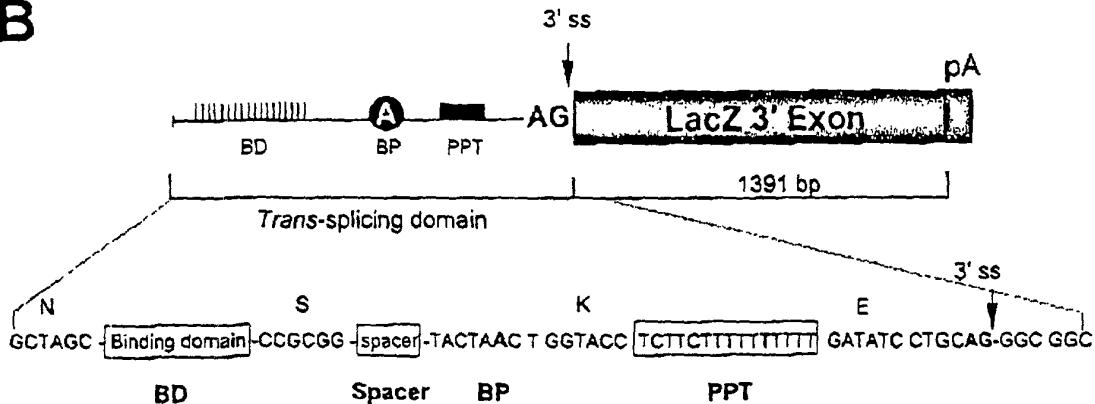


A

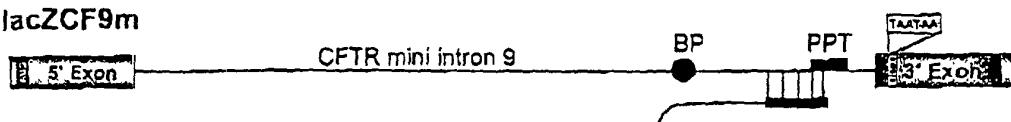
Figure 37 A

*Sheet 45 of 58*

B



*lacZCF9m*



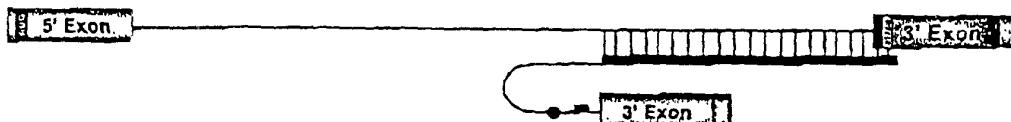
PTM-CF14  
23 bp BD



PTM-CF22  
91 bp BD



PTM-CF24  
153 bp BD



PTM-CF26  
200 bp BD



PTM-CF27  
411 bp BD

*Figure 37B*

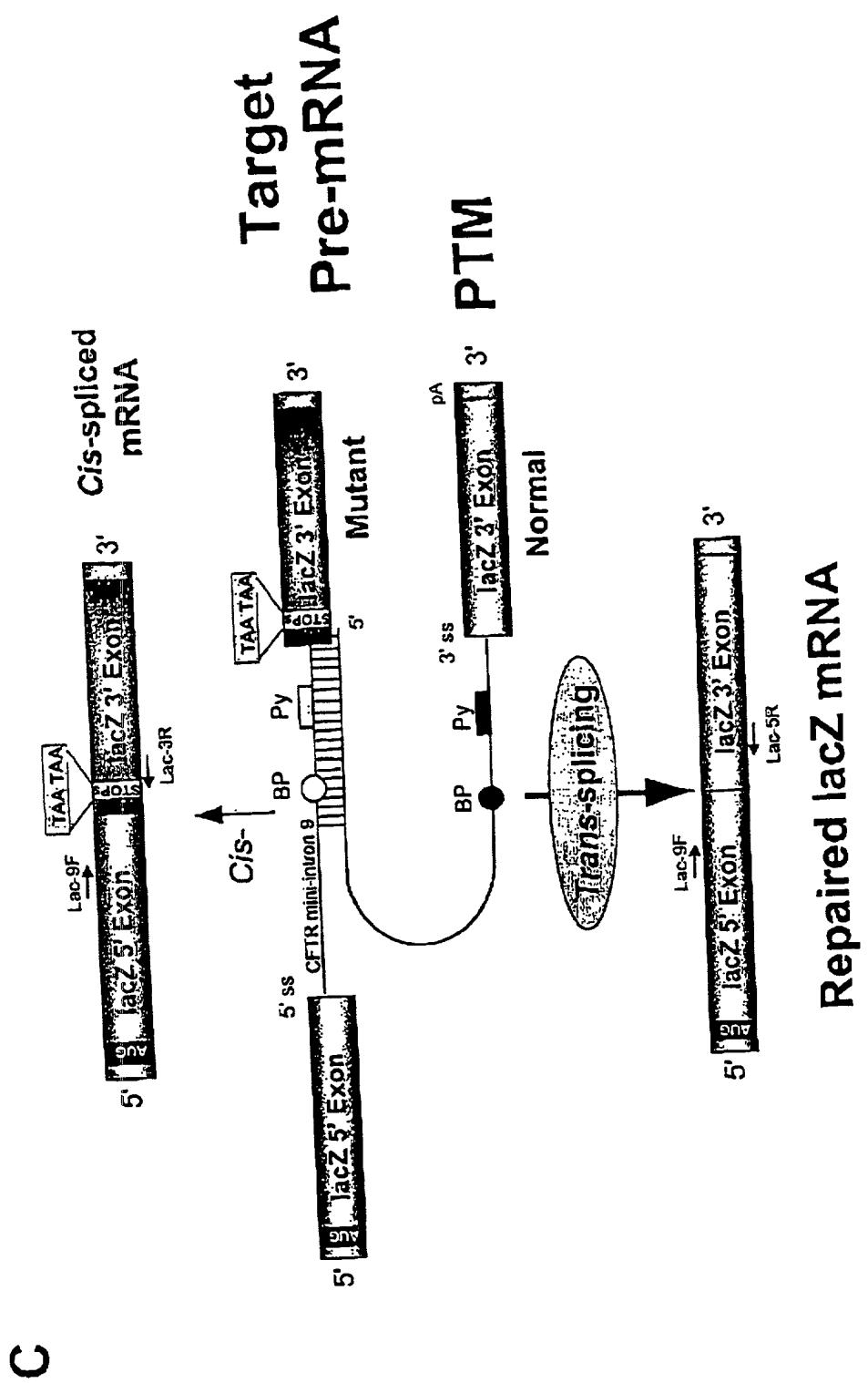


Figure 37C

about 46 of 58

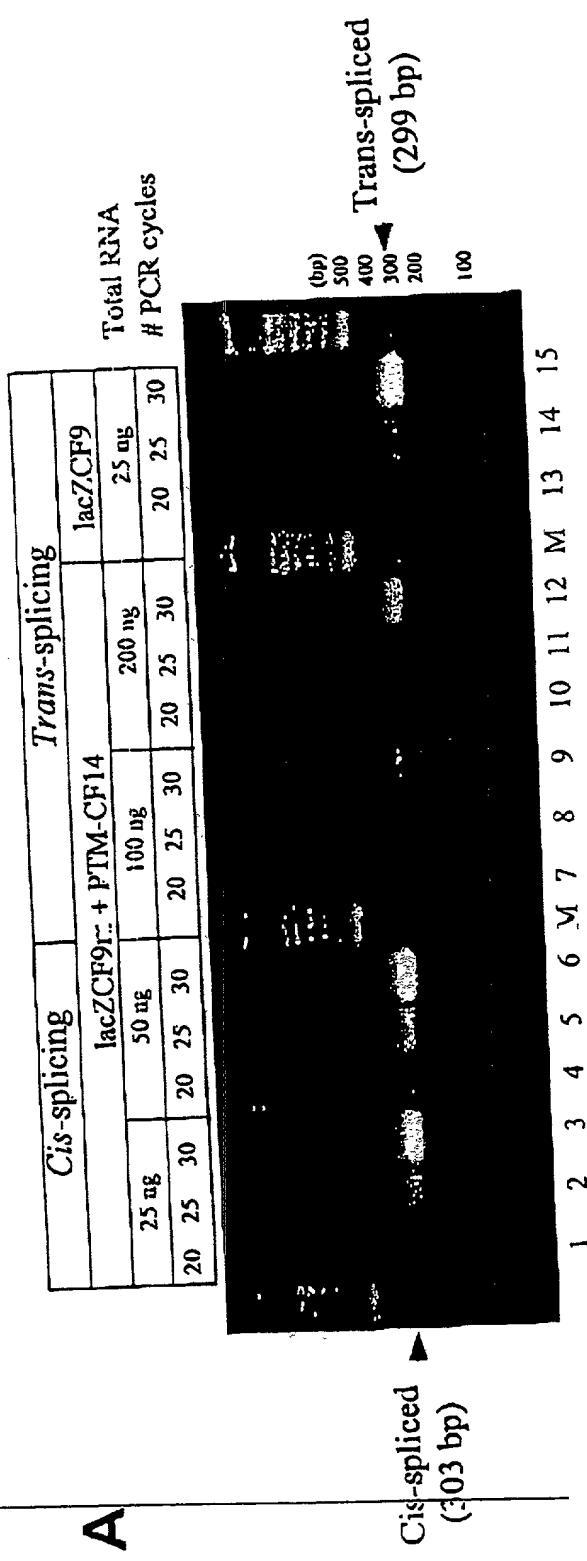
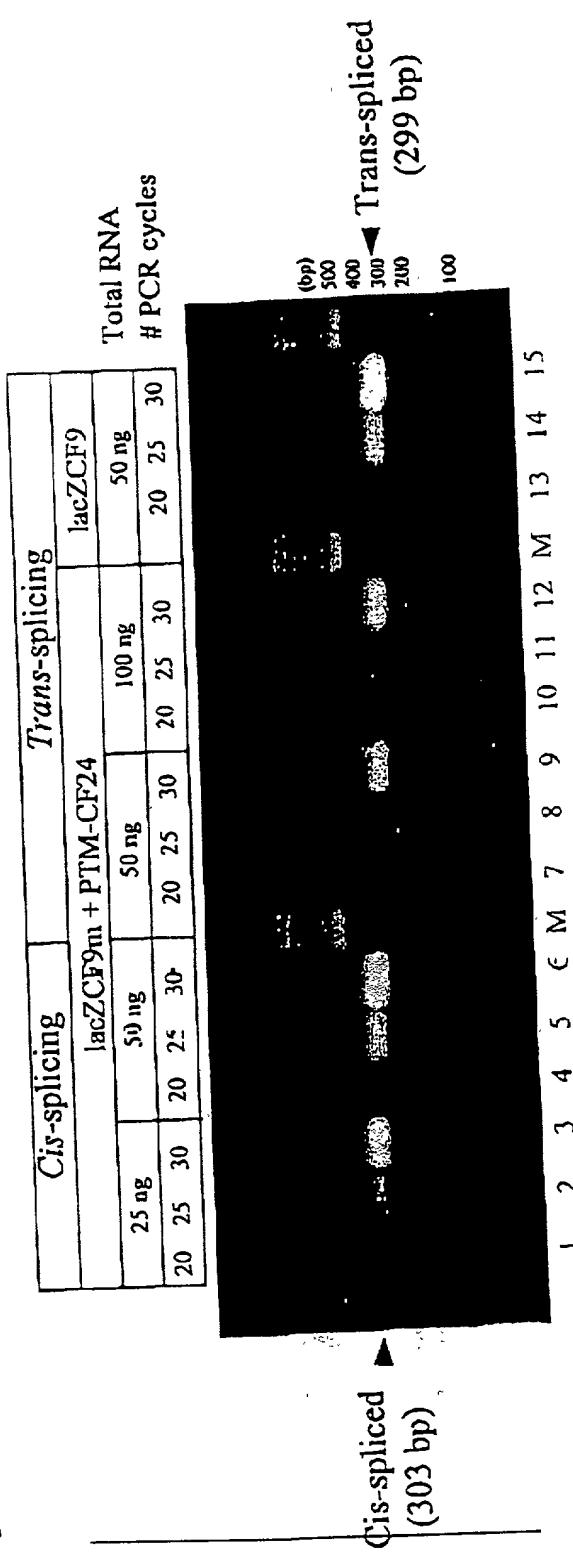


Figure 38 A



85 for th tmyd

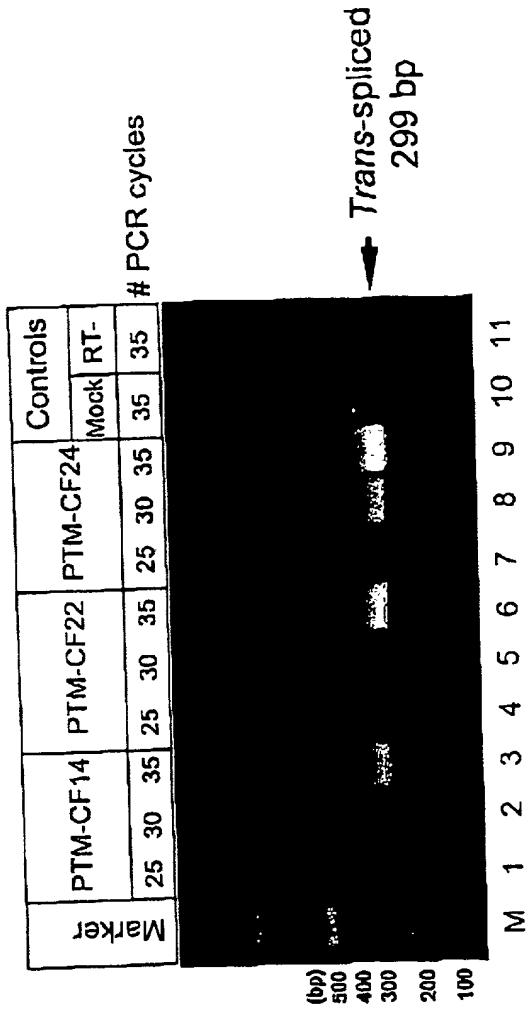
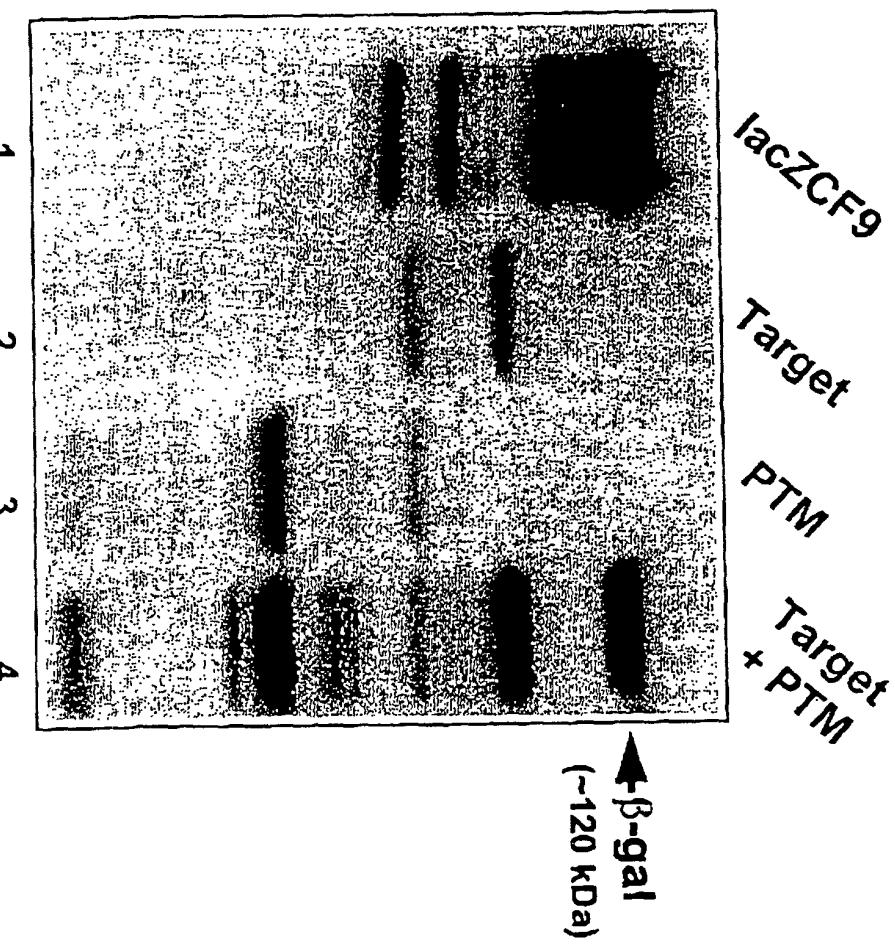
**B**

Figure 38B

Adult 48 of 58

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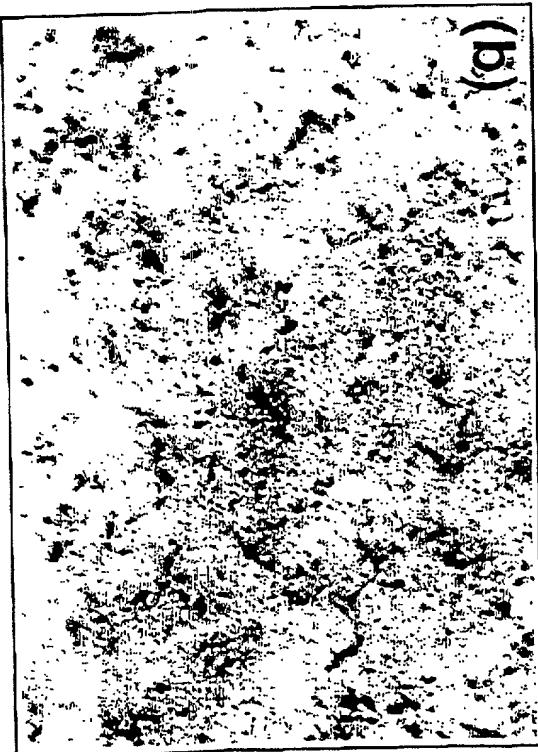
Figure 39



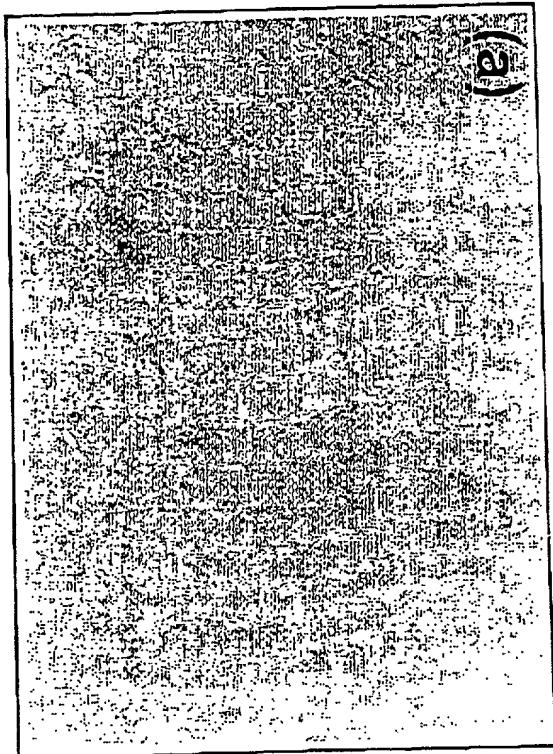
Sheet 50 of 58

Figure 40A

A



(b)



(a)

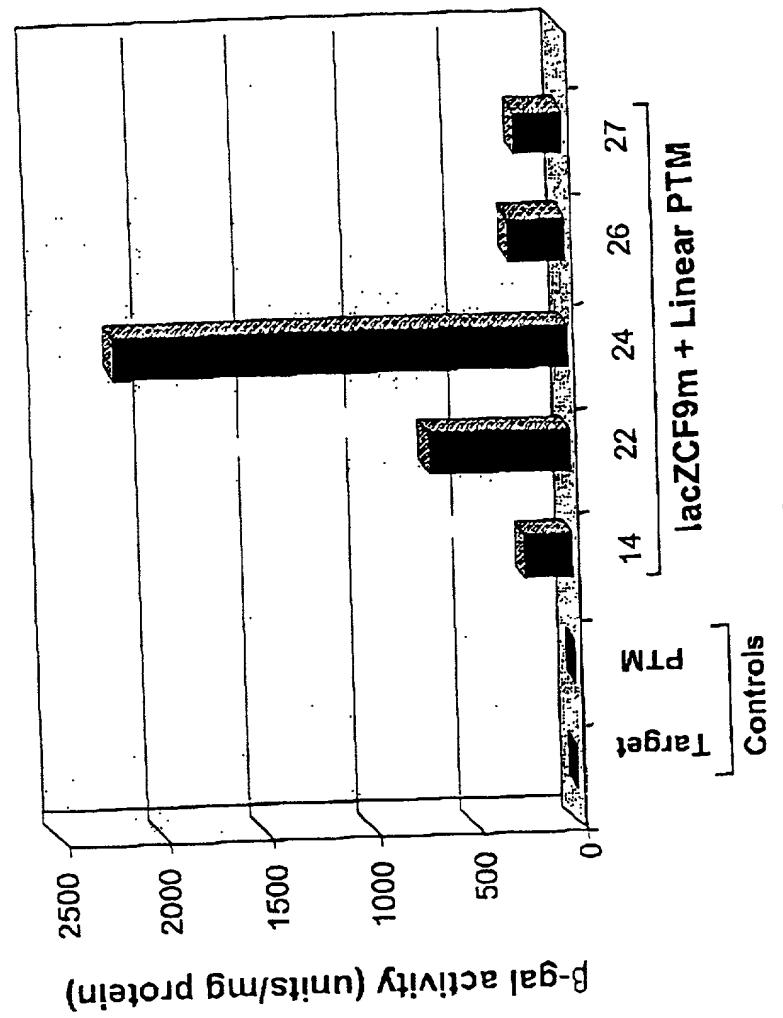
**B**

Figure 40B

*adult SI of 58*

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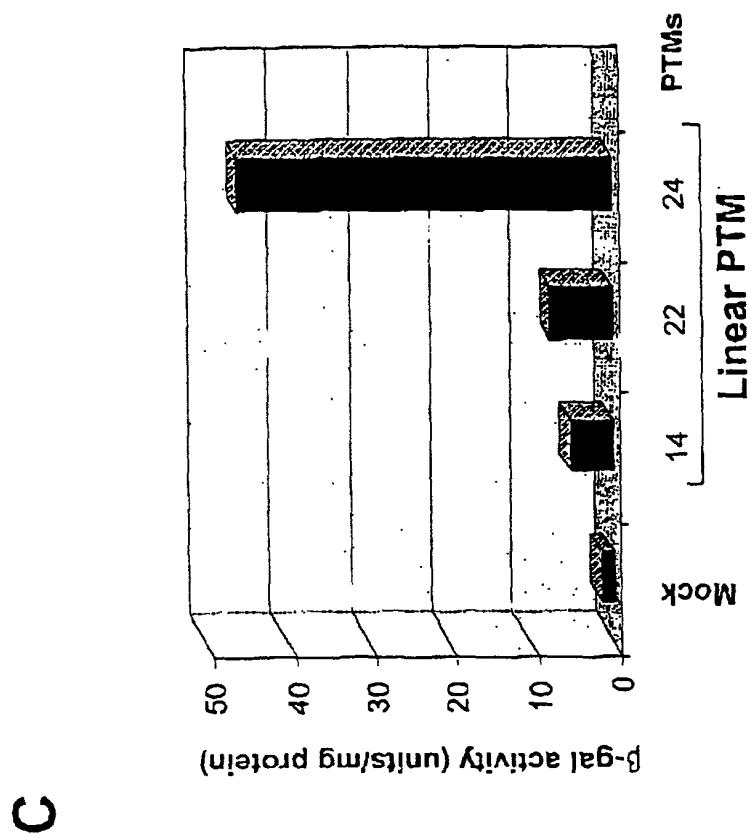


Figure 40C

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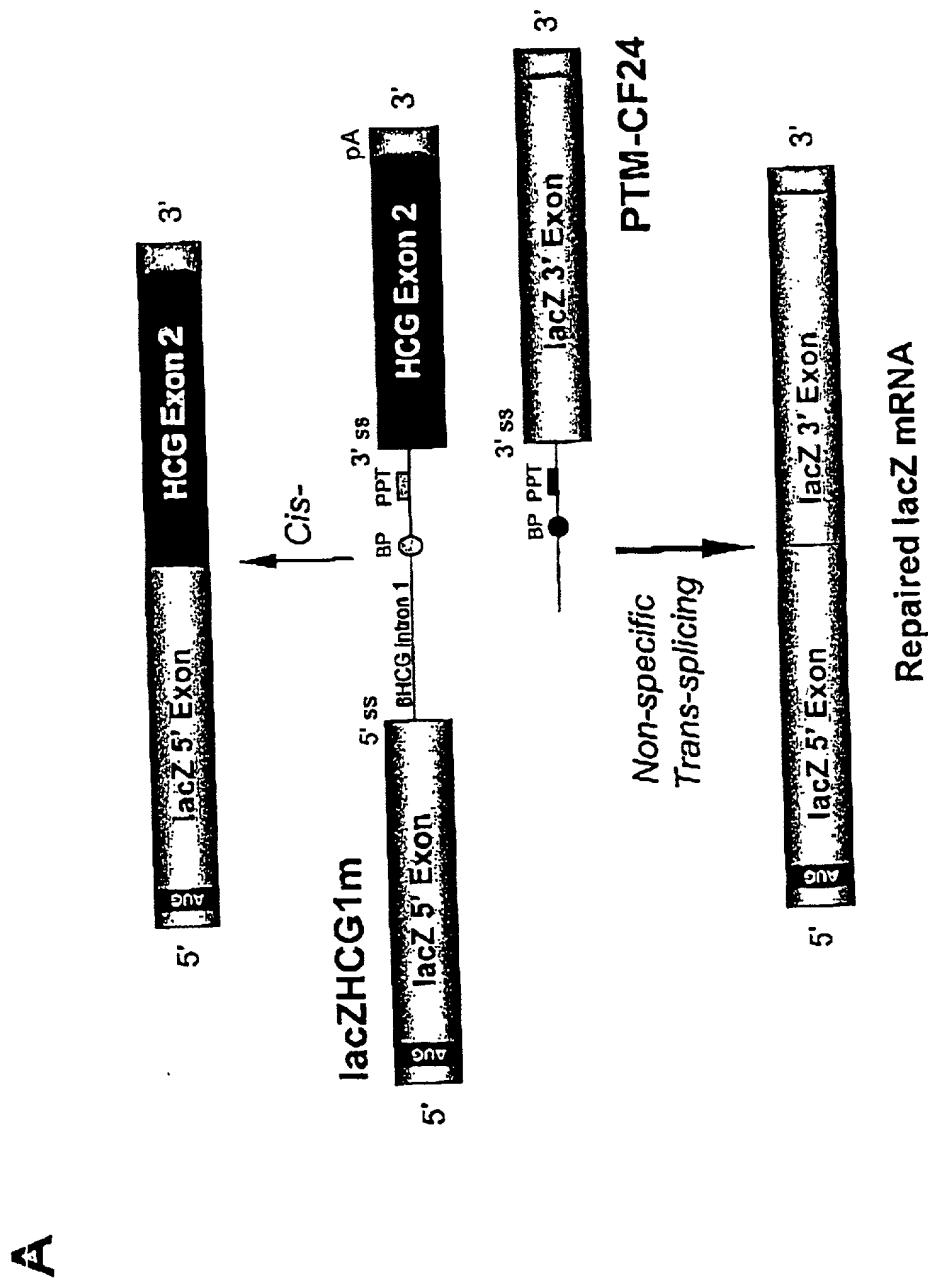


Figure 41A

Sheet 54 of 58

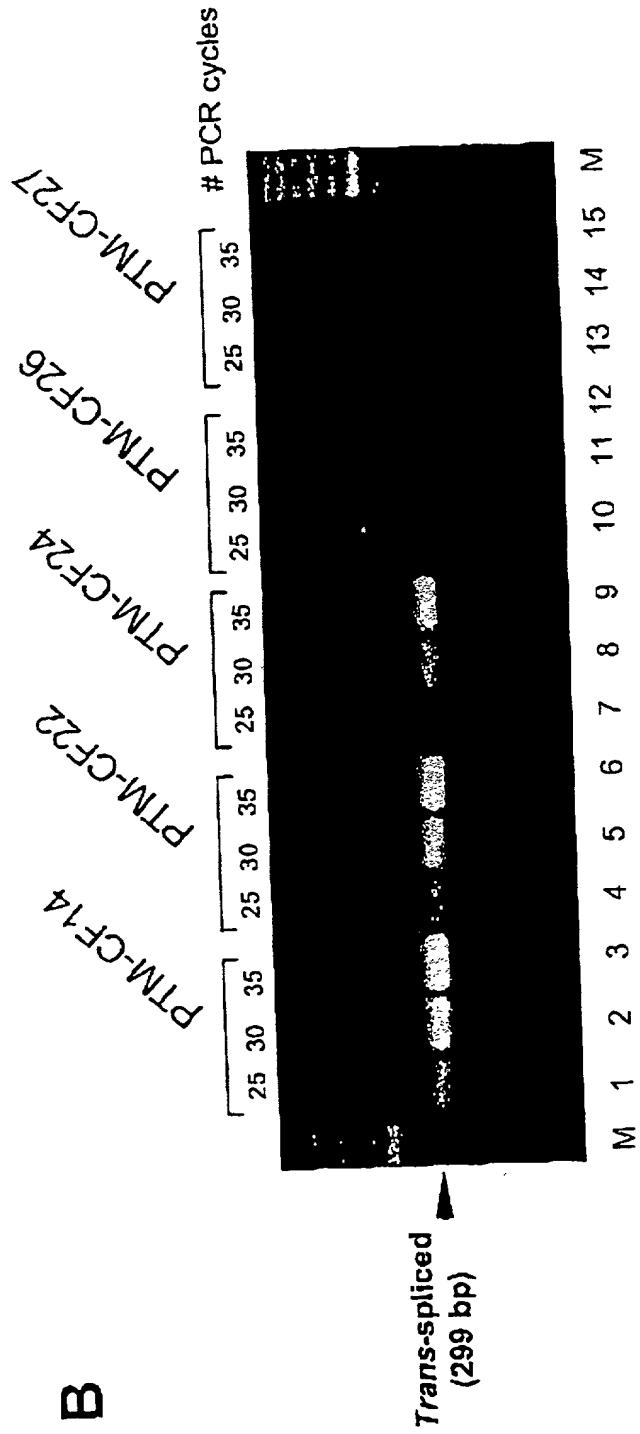


Figure 4(B)

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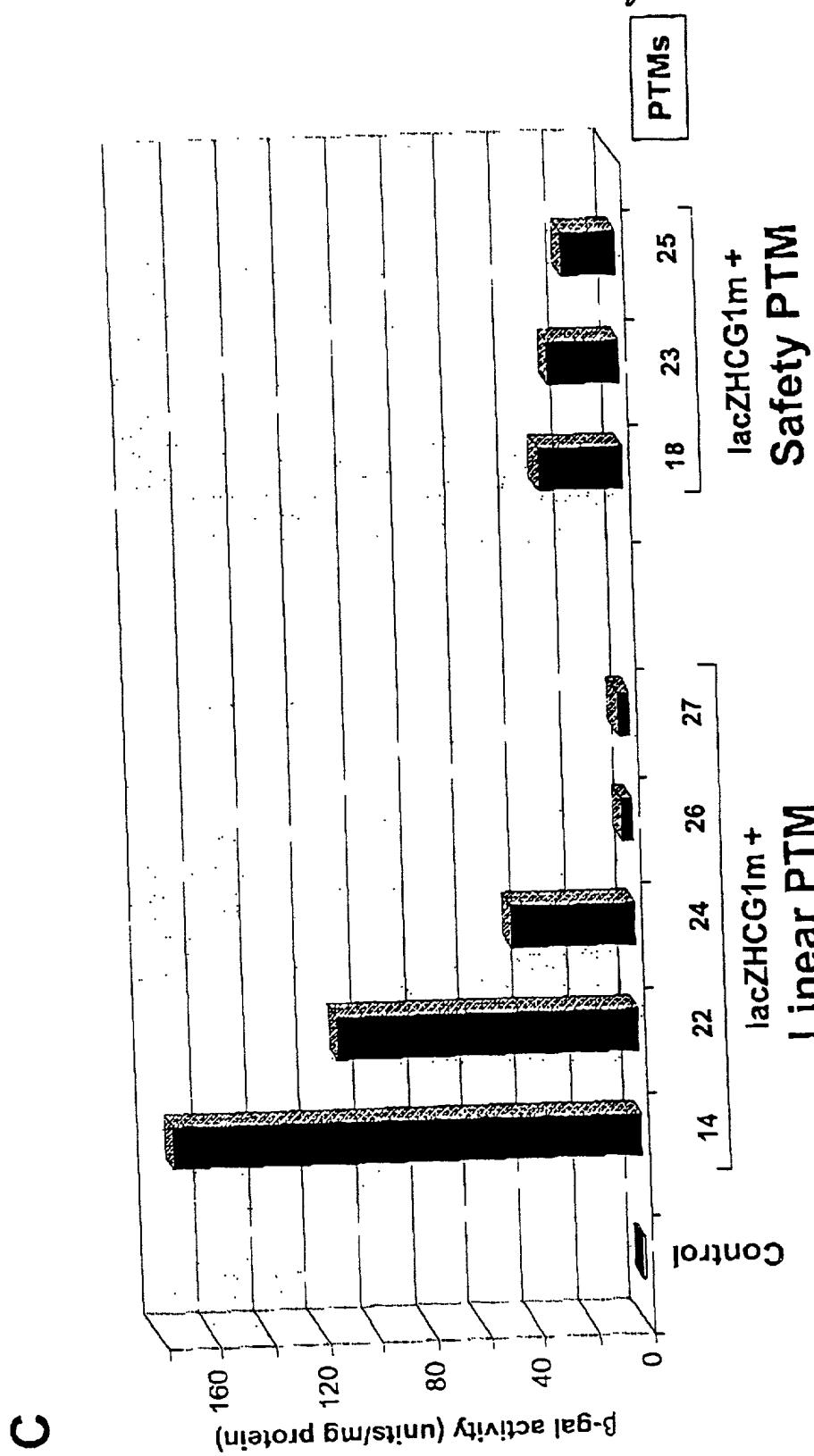


Figure 4C

*Sheet 56 of 58*

Exons 1-10

ATGCAGAGTCGCCCTGGAAAAGGCCAGCGTTCTCAAACCTTTTCAGCTGGACCAGACCAATTGAGGAAAG  
GATACAGACAGCGCCCTGGAATTGTCAGACATATAACCAATCCCTCTGTTGATTCTGCTGACAATCTATCTGAAAAATT  
GGAAAGAGAATGGGATAGAGAGCTGGCTTCAAAGAAAATCTAAACTCATTAATGCCCTCGGCATGTTTTCTGG  
AGATTATGTTCTATGGAATCTTTTATATTAGGGGAAGTCACCAAAGCAGTACAGCCTCTTACTGGGAAGAATCA  
TAGCTCCTATGCCCGATAACAAGGAGGAACGCTCATCGCGATTATCTAGGCATAGGCTATGCCCTCTCTTTAT  
TGTGAGGACACTGCTCCTACACCCAGCCATTGCGCTTCATCACATTGGAATGCGAGATGAGAATAGCTATGTTAGT  
TTGATTATAAGAAGACTTTAACGCTGTCAGCGCTGTTCTAGATAAAAGTATTGACAACCTGTTAGTCTCCCTT  
CCAACAACTGAAACAAATTGATGAGGACTTGCACTTGCATTCGCTGGATCGCTCTTGCAAGTGGCACTCCT  
CATGGGCTAATCTGGAGTTGTTACAGGGCTCTGCGCTCTGCGACTTGGTTCTCTGATAGTCTTGCCCTTTTCAG  
GCTGGGCTAGGGAGAATGATGAGTCAAGAGATCAGAGACTGGGAAGATCAGTGAAGAGACTTGTGATTACCTCAG  
AAATGATCGAGAACATCCAATCTGTTAAGGCATACTGCTGGGAAGAGCAATGGAAAAATGATTGAAAACCTTAAGACA  
AACAGAACTGAAACTGACTCGAAGGCAGCCTATGTGAGATACTTCATAGCTCAGCCTCTTCTCAGGGTTCTT  
GTGGTGTCTTATCTGCTCCCTATGCACTAATCAAGGAATCATCCTCGGAAATATTCAACCACATCTCATTCT  
GCATTGTTCTGCCATGGCGGTCACTCGGCAATTCCCTGGGCTGACAAACATGGTATGACTCTCTGGAGCAATAAA  
CAAATACAGGATTCTTACAAAAGCAAGAATATAAGACATTGGAATATAACTTAACGACTACAGAAGTAGTGTGGAG  
AATGTAACAGCCTCTGGGAGGGATTGGGAATTATTGAGAAAGCAAAACAAAACAATAACAATAGAAAAACTT  
CTAATGGTGTGACAGCCTCTTCTCAGTAATTCTCAGTCTGGTACTCCTGCTGAAAGATATTCAAGAT  
AGAAAGAGGACAGTTGTTGGCGGTGCTGGATCCACTGGAGCAGGCAAGACGAGCTTGCTCATGATGATCATGGCGAG  
**TTAGAACCAAGTGAAGGCAAGATCAAACATTCCGGCCGATCAGCTTGTGAGCCAATTCAAGTGGATCATGCCCGTA**  
**CCATCAAGGAGAACATAATCTCGGCGTCAAGTTACGACGAGTACCGCTATCGCTCGGTGATTAAGGCCTGTCAGTTGGA**  
**GGAG**

Trans-splicing domain

**GTAAGATATCACCGATATGTCATAACCTGATTCGGGCCCTCGATACGCTAACGATCCACCGG**  
**TCAAAAAGTTTACATAATTCTTACCTCTTGAATTCTGCTTGTATGACGCTTGTATCTATATTCTCATTG**  
**GAAACACCAATGATATTCTTAATGGTGCCTGGCATAATCCTGGAAAATCTGATAACACAATGAAAATTCTCCACTGT**  
**GCTTAATTCTACCTCTGAATTCTCCATTCTCCATAATCATCATACAACGAACTCTGAAATTAAACCATCATT**  
**ATTAACCTCATTCAAATCACGCT**

Figure 42

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153 bp PTM24 Binding Domain:

Nhe I                    153 bp BD underlined  
**GCTAGC**-ATAATGACGAAGCCGCCCTCACCGCTCAATTATCATCCTAAGCAGAAGTGTATA  
TTCTTATTGTAAAGATTCTTAACTCATTTGANTTAAATAATTAAATAACTTCCTGGTTTCACTTCTGCTATGC

Sac II

Figure 43A

*Sheet 58 of 58*

Trans-splicing domain

AATAATGACGAAGCCGCCCTCACGCTCAGGATTCACTTGCCCTCCAATTATCATCCTAAGCAGAAGTGTATATTCTTA  
TTTGTAAAGATTCTATTAACTCATTGATCAAAATATTAAAATACTTCCTGTTCACCTACTCTGCTATGCACCCGC  
GGAACATTATTATAACGTTGCTGAATACTAAGTGTACCTCTTCTTTTTGATATCCTGCAG

Exons 10-24

ACTTCACTTCTAATGATGATTATGGGAGAACTGGAGCCTCAGAGGGTAAAATTAAAGCACAGTGGAGAATTTCATTCT  
GTTCTCAGTTTCTGGATTATGCCTGGCACCTAAAGAAAATATCATCTTGGTGTTCCTATGATGAATATAGATA  
CAGAACGCGTCAAAAGCATGCCAAGTAGAAGAGGACATCTCAAGTTGCAGAGAAAAGACAATATAGTCTGGAGAA  
GGTGGAACTCACACTGAGTGGAGGTCAACGAGCAAGAATTCTTAGCAAGAGCAGTATAACAAAGATGCTGATTGTATT  
TATTAGACTCTCTTTGGATACCTAGATGTTTAACAGAAAATATTGAAAGCTGAACTTAAAGGAAAGCTGACAAATATTAATTTGATGAGG  
TAACAAAATAGGATTTGGTCACTCTAAAGGAAATGGAACTTCAACCTCAACTGAGACCTAACCGTTCTCATTAGAAGGAGATGC  
AGCAGCTATTTTATGGGACATTTCAAGAACTCCAAATCTACAGCCAGACTTAGCTCAAAACTCATGGATGTT  
CTTCGACCAATTAGTGCAGAAAGAAGAAATTCAACCTCAACTGAGACCTAACCGTTCTCATTAGAAGGAGATGC  
TCCTGTCCTGGACAGAAACAAAAACATCTTAAACAGACTGGAGAGTTGGGAAAAAGGAAGAATTCTATT  
CTCAATCCAATCAACTCTATAAGAAATTTCATTGTGCAAAGACTCCCTACAAATGAATGGCATCGAAGGAGATT  
CTGATGAGCCTTAGAGAGAAGGCTGCTTAGTACAGATTCTGAGCAGGGAGAGGCGATACTGCCTCGCATCAGCGT  
GATCAGCACTGGCCCCACGCTCAGGCACGAAGGAGGAGTCTGCTGAACCTGATGACACACTCAGTTAACCAAGGT  
CAGAACATTCAACGAAAGACACAGCATCCACACGAAAAGTGTACTGGCCCTCAGGCAAATTGACTGAACGGATA  
TATATTCAAGAAGGTTATCTCAAGAAAATGGCTTGGAAATAAGTGAAGAAAATTAAAGAAGAAGCTTAAAGGAGTGTCTT  
TTTGATGATATGGAGAGCATACCAGCAGTGAACATGGAAACACATACCTTCGATATATTACTGTCCACAGAGCTTA  
ATTTTGTGCTAATTGGCTTAGTAAATTCTGTCAGGGCTGCTTGGCTGTGCTGGCTCTGGAA  
ACACTCTCAATTCAAGTGTGAAATTTTACACCACAAAATGTTACATTCTGTCAGGGCTTCAAGCACCTATGTCAACCCCTA  
ACACGTTGAAAGCAGGTGGGATTCTTAATAGATTCTCAAAGATAAGCAATTGGATGACCTCTGCTTCTTACCCAGCACCAGTTC  
ATTTGACTTCATCAGTTGTTATTGATGGCTATAGCAGTTGTCAGTTTACAACCTACATCTTGT  
GCAACAGTGCCAGTGATGTGCTTTTATTATGTTGAGAGCATATTCTCAAACCTCACAGCAACTCAACAACTGG  
AATCTGAAGGCAGGAGTCAAATTTCACTCATCTGTTACAAGCTTAAAGGACTATGGACACTTCGTGCCCGACG  
GCAGCCTACTTGAACACTGTTCCACAAAGCTCTGAATTACACTGCCAACGGTTCTGTACCTGTCAACACTG  
CGCTGTTCCAATGAGAATAGAAATGATTGTCATCTTCTCATTGCTTACCTTCATTCCATTAAACACAG  
GAGAAGGAGAAGGAAGAGTGTGATTATCTGACTTTAGGCCATGAATATCATGAGTACATTGCCAGTGGCTGTAAACCTC  
CAGCATAGATGTGATAGCTGATGCGATCTGAGGCCAGTCTTAAGTTCATTGACATGCCAACAGAAGGTAACCT  
ACCAAGTCAACCAACCATAACAAGAATGCCAACCTCTGAAGTATGATTAGAGAAATTACACGTGAAGAAAGATG  
ACATCTGGCCCTCAGGGGCCAAATGACTCTCAAAGATCTCACAGAAAATACACAGAAGGTGGAAATGCCATTAGA  
GAACATTTCCTCTCAATAAGTCTGCCAGAGGGTGGCCTTGGGAAGAACTGGATCAGGGAGAGTACTTTGTTA  
TCAGCTTTTGAGACTACTGAACACTGAAGGAGAAATCCAGATGGTGTCTGGGATTCAATAACTTTGAAAC  
AGTGGAGGAAAGCCTTGAGTGATACCAAGAGAATTCTGAACTTGGGATTCTGAACTTGGGATTCTGAACTTGGG  
AAGCTTGAATTGCTCTGAGATGGGCTGCTCTGCTTAAGCCATGCCAACAGCAGTTGATGCTTGGCTAGATCTG  
TTCTCAGTAAGGCCAGATCTGCTCTGATGAACTGCCAACAGTGTCTTGGATCCAGTAACACATACCAATAATTAGAAG  
AACTCTAAACAAAGCATTGCTGATGGCAGTACGTAATTCTCTGTGAACACAGGATAGAAGCAATGCTGAAATGCCAACAA  
TTTTGGTCAAGAGAGAACAAAGTGCAGTACGTTCCAGAAACTGCTGAACAGGAGAGGCCCTTCCGGC  
AAGCCATCAGCCCCCTCCGACAGGGTGAAGCTCTTCCCCACCGAACAGCAAGTGTCAAGCCCCAGATTGC

Histidine tag Stop

TGCTCTGAAAGAGGAGACAGAAGAAGAGGTGCAAGATAAGGCTTCATCATCATCATCATTAG

Figure 43B